

Szkoła Doktorska Nauk Ścisłych i Przyrodniczych
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**Ocena fitotoksyczności i selektywności olejku
tatarakowego oraz ustalenie docelowych miejsc
jego oddziaływania w korzeniach zarodkowych
dwóch gatunków uprawnych (*Vicia faba* L.
var. minor i *Lupinus luteus* L.) oraz segetalnych
(*Brassica napus* L. i *Arabidopsis thaliana* L.)**

Assessment of the phytotoxicity and selectivity of calamus essential oil and
identification of its target sites of action in primary roots of two cultivated
species (*Vicia faba* L. *var. minor* and *Lupinus luteus* L.) and two weed species
(*Brassica napus* L. and *Arabidopsis thaliana* L.)

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1. Źródła finansowania badań



Projekt Ocena fitotoksyczności i selektywności olejku tatarakowego oraz ustalenie docelowych miejsc jego oddziaływania w nasionach i siewkach dwóch gatunków uprawnych (*Vicia faba* L. var. *minor* i *Lupinus luteus* L.) oraz segetalnych (*Brassica napus* L. i *Arabidopsis thaliana* L.) finansowany w ramach konkursu Doktoranckie Granty Badawcze, finansowanych z subwencji IDUB dla Uniwersytetu Łódzkiego, 9/DGB/IDUB/2022



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2. Współpraca

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2. **Wróblewski, M.***; Piotrowska-Niczyporuk, A.; Ciereszko, I.; Gocek, N.; Żabka, A.; Szczepblewski, P.; Sobiech, Ł.; Saja-Garbarz, D.; Polit, J. T. Phytotoxicity and Bioherbicidal Potential of Sweet Flag (*Acorus calamus* L.) Essential Oil on Fabaceae and Brassicaceae Species. *Scientia Horticulturae* **2025**, 347, 114180. <https://doi.org/10.1016/j.scienta.2025.114180>.
3. **Wróblewski, M.***; Krajewski, K.; Gocek, N.; Żabka, A.; Polit, J. T. *Acorus calamus* L. Essential Oil Induces Oxidative Stress and DNA Replication Disruptions in Root Meristem Cells of Two Fabaceae and Two Brassicaceae Species. *IJMS* **2025**, 26 (10), 4715. <https://doi.org/10.3390/ijms26104715>.
4. **Wróblewski, M.**; Gocek, N.; Żabka, A.; Polit, J. T.* Mitotic Disruption and Cytoskeletal Alterations Induced by *Acorus calamus* Essential Oil: Implications for Bioherbicidal Potential. *IJMS*. **2025**, 26 (18), 8933. <https://doi.org/10.3390/ijms26188933>.
5. **Wróblewski, M.**; Gocek-Szczerpek, N.; Krajewski, K.; Żabka, A.; Polit, J. T.* Ultrastructural, signaling, and epigenetic responses to *Acorus calamus* L. essential oil in root meristems of Fabaceae and Brassicaceae seedlings – manuskrypt wysłany do czasopisma *The Plant Journal*

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Summaryczny współczynnik wpływu (ang. *Impact Factor*, IF) opublikowanych prac wchodzących w skład rozprawy doktorskiej (1-4): **IF = 17,8**

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4. Streszczenie

Rosnące ograniczenia stosowania herbicydów syntetycznych nasilają poszukiwania zrównoważonych alternatyw pochodzenia roślinnego. W niniejszej pracy, łącząc podejścia biochemiczne, cytologiczne i molekularne, oceniono efekty fitotoksyczne, selektywność oraz mechanizm działania olejku eterycznego z kłączy tataraku zwyczajnego (*Acorus calamus* L., SEO). Analiza składu olejku wykazała dominację fenylopropanoidów, w tym β -azaronu, a także innych związków fenylopropanowych i seskwiterpenów. Dla zapewnienia porównywalnej siły stresu ustalono gatunkowo specyficzne stężenia (IC_{50}) hamujące o połowę wzrost korzeni zarodkowych: dla dwóch gatunków Fabaceae (*Vicia faba*: 0,03%; *Lupinus luteus*: 0,025%) oraz dwóch gatunków Brassicaceae (*Brassica napus*: 0,01%; *Arabidopsis thaliana*: 0,005%). 24-godzinna ekspozycja na zemulgowany SEO indukowała spadek świeżej i suchej masy korzeni, uszkodzenia błon komórkowych i wyciek elektrolitów oraz obniżenie żywotności komórek związane ze wzrostem poziomu reaktywnych form tlenu (ROS). Pomimo aktywacji kompleksowej enzymatycznej i nieenzymatycznej odpowiedzi antyoksydacyjnej, stwierdzono peroksydację lipidów oraz zmiany metaboliczne potwierdzone zarówno kalorymetrią izotermiczną, jak i analizą zawartości białek, cukrów i kwasów tłuszczowych. Ogólny wzorzec reakcji wskazał na większą odporność przedstawicieli Fabaceae, podczas gdy Brassicaceae wykazały słabszą adaptację metaboliczną i silniejsze uszkodzenia. Wzrost korzeni zarodkowych zależy od proliferacji w merystemie wierzchołkowym, dlatego przeanalizowano wpływ SEO na aktywność komórek merystematycznych. ROS akumulowały się tu głównie w organellach prowadząc do uszkodzenia błon, zahamowania aktywności heksokinazy (HXK) i aktywacji szlaku MAPK (p44/42), który przenosił sygnał stresu do jądra. Indukowały także pęknięcia DNA i stres replikacyjny. W efekcie obserwowano remodeling chromatyny, hipermetylację DNA, utratę znaczników epigenetycznych związanych z aktywną transkrypcją, spadek syntezy RNA, wakuolizację jąderka, a jednocześnie powstanie ognisk epigenetycznych wskazujących na aktywację genów stresowych. Nastąpiło też nagromadzenie komórek w fazie G1 oraz spadek liczby komórek replikujących DNA (inkorporujących 5-etyniło-2'-deoksyurydynę, EdU), a także wydłużenie fazy S, szczególnie w końcowym etapie syntezy skondensowanej heterochromatyny. Zjawiskom tym towarzyszyła aktywacja mechanizmów naprawczych, potwierdzona obecnością sygnałów γ -H2AX i specyficznych modyfikacji histonowych. Pomimo stresu genotoksycznego, SEO nie indukował rozległej fragmentacji DNA ani przełączenia cyklu na endocykl. Redukował natomiast liczbę komórek mitotycznych, a wśród dzielących się indukował akumulację w metafazie i powstanie aberracji chromosomowych, powiązanych z nadmierną stabilizacją włókien cytoszkieletu, utrudniającą reorganizację wrzeciona kariokinetycznego i fragmoplastu. Zaburzał też regulację epigenetyczną mitozy poprzez ingerencję w molekularne mechanizmy kontrolne obejmujące kinazę Cdc2 i fosfatazy PP1/2A. Porównania międzygatunkowe wykazały większą plastyczność chromatyny i sprawniejsze mechanizmy naprawcze u Fabaceae, podczas gdy Brassicaceae cechowały się silniejszą represją transkrypcji i stresem replikacyjnym. Łącznie uzyskane wyniki dowodzą, że SEO działa poprzez złożony, wielotorowy mechanizm, obejmujący jednocześnie zaburzenia błon, metabolizmu, kaskad sygnalizacyjnych, organizacji chromatyny i dynamiki cytoszkieletu. Systemowy charakter tych oddziaływań tłumaczy wyraźną selektywność między rodzinami roślin i ogranicza ryzyko ewolucji odporności. Integracja selektywności fitotoksycznej z wielopoziomowym zakłócaniem procesów komórkowych czyni z SEO obiecującego kandydata na bioherbicyd w systemach rolnictwa zrównoważonego.

5. Abstract

Growing restrictions on the use of synthetic herbicides intensify the search for sustainable plant-derived alternatives. In this study, by combining biochemical, cytological, and molecular approaches, we assessed the phytotoxic effects, selectivity, and mechanism of action of essential oil from rhizomes of sweet flag (*Acorus calamus* L., SEO). Oil composition analysis revealed the dominance of phenylpropanoids, mainly β -asarone, along with other phenylpropanoids and sesquiterpenes. To ensure comparable stress intensity, species-specific half-inhibitory concentrations (IC_{50}) for root growth were established: for two Fabaceae (*Vicia faba*: 0.03%; *Lupinus luteus*: 0.025%) and two Brassicaceae (*Brassica napus*: 0.01%; *Arabidopsis thaliana*: 0.005%). A 24-h exposure to emulsified SEO reduced fresh and dry root biomass, impaired membrane integrity, increased electrolyte leakage, and decreased cell viability due to elevated reactive oxygen species (ROS) levels. Despite activation of both enzymatic and non-enzymatic antioxidant responses, lipid peroxidation was detected, and metabolic changes were revealed by isothermal calorimetry and analysis of proteins, sugars, and fatty acids. The general response pattern indicated higher resistance in Fabaceae, whereas Brassicaceae showed weaker metabolic adjustment and stronger damage. Since root growth depends on meristematic proliferation, we next analyzed the impact of SEO on meristem activity. ROS accumulated mainly in organelles, causing membrane damage, inhibition of hexokinase (HXK), and activation of the MAPK (p44/42) pathway, transmitting stress signals to the nucleus. They also induced DNA breaks and replication stress. As a consequence, chromatin remodeling, DNA hypermethylation, loss of active transcription marks, reduced RNA synthesis, nucleolar vacuolization, and stress-related epigenetic foci were observed. Cells accumulated in G1 phase, while the number of replicating cells (EdU-incorporating) declined and S phase was prolonged, particularly during replication of condensed heterochromatin. These effects were accompanied by activation of repair mechanisms, confirmed by γ -H2AX signals and specific histone modifications. Despite genotoxic stress, SEO did not induce extensive DNA fragmentation or switch to endocycling. However, it reduced the number of mitotic cells, and in dividing cells caused metaphase arrest and chromosome aberrations, associated with excessive stabilization of cytoskeletal fibers, impairing spindle and phragmoplast reorganization. SEO also disrupted epigenetic regulation of mitosis through interference with control mechanisms involving Cdc2 kinase and PP1/2A phosphatases. Comparative analyses showed greater chromatin plasticity and more efficient repair mechanisms in Fabaceae, whereas Brassicaceae exhibited stronger transcriptional repression and replication stress. Altogether, the results demonstrate that SEO acts through a complex, multitarget mechanism involving simultaneous disruption of membranes, metabolism, signaling cascades, chromatin organization, and cytoskeletal dynamics. This systemic mode of action explains the clear interfamily selectivity and reduces the risk of resistance evolution. Integration of phytotoxic selectivity with multilayered cellular disruption makes SEO a promising candidate for bioherbicide development in sustainable agriculture.

6. Omówienie celu naukowego rozprawy i uzyskanych wyników

6.1. Wprowadzenie

Wprowadzenie syntetycznych środków ochrony roślin na początku XX wieku było momentem przełomowym zarówno dla rolnictwa, jak i dla całej ludzkości. Preparaty te obejmują szeroką grupę związków chemicznych o działaniu toksycznym wobec różnych organizmów, w tym chwastów, patogenów i owadów. Najczęściej określa się je wspólnym mianem „pestycydów”, wśród których wyróżnia się przede wszystkim herbicydy, fungicydy i insektycydy. Ich masowe stosowanie przyczyniło się do ograniczenia strat w produkcji roślinnej, zwiększenia plonów oraz poprawy jakości żywności, przy jednoczesnym obniżeniu kosztów wytwarzania. Proces ten odegrał znaczącą rolę w zwiększeniu dostępności żywności i w redukcji problemu głodu oraz niedożywienia w wielu regionach świata [1–4].

Choć pestycydy odegrały znaczącą rolę w rozwoju rolnictwa, z czasem ujawniły się także ich poważne konsekwencje zdrowotne i środowiskowe. Związki te przedostają się do ekosystemu głównie w wyniku zabiegów agrotechnicznych, a następnie mogą akumulować się w glebie i przenikać do wód gruntowych [5]. W organizmie człowieka oddziałują niekorzystnie na skórę, układ oddechowy, pokarmowy, nerwowy i rozrodczy przyczyniając się m.in. do zaburzeń hormonalnych, a także zwiększając ryzyko rozwoju nowotworów [1]. Ich chemiczna stabilność utrudnia rozkład przez mikroorganizmy, sprzyjając długotrwałej obecności w środowisku. Nieracjonalne i intensywne stosowanie pestycydów doprowadziło również do jednego z poważniejszych problemów ostatnich dekad, a mianowicie gwałtownie narastającej odporności u zwalczanych organizmów, wymuszając tym samym zwiększanie dawek, a w konsekwencji wycofywanie wielu substancji czynnych z rynku i zastępowanie je kolejnymi równie toksycznymi [6]. Mechanizmy nabywanej odporności dzielą się na dwa główne typy: odporność w miejscu docelowym (ang. *target site resistance*, TSR) oraz odporność poza miejscem docelowym (ang. *non-target site resistance*, NTSR). TSR wynika z mutacji w obrębie genów kodujących białka docelowe, przez co substancja czynna przestaje zakłócać ich funkcję i nie prowadzi do śmierci organizmu. Natomiast NTSR obejmuje różnorodne mechanizmy fizjologiczne i komórkowe, takie jak intensyfikacja metabolizmu herbicydów, ograniczenie ich poboru, zmiana kierunku transportu czy sekwestracja związku np. w wakuoli [7]. Podobne strategie adaptacyjne obserwuje się zarówno u chwastów jak i u patogenów i owadów, co dodatkowo komplikuje skuteczne ograniczanie ich liczebności i zwiększa skalę problemu.

Obecnie na świecie stosuje się rocznie około 2 mln ton pestycydów, a prognozy wskazują, że do 2030 r. ilość ta może wzrosnąć do 4,5 mln ton [8]. W odpowiedzi na te wyzwania Unia Europejska podjęła działania zmierzające do ograniczenia szkód wynikających z nadmiernego stosowania pestycydów oraz do zapewnienia produkcji żywności w sposób zrównoważony. Aktualne plany zakładają redukcję zużycia syntetycznych pestycydów o 50% do 2030 r. oraz stopniowe zastępowanie ich preparatami pochodzenia naturalnego, które mogą wspierać lub nawet całkowicie zastąpić środki chemiczne [9].

Poza zagrożeniami ze strony patogenów i szkodników, istotnym wyzwaniem w ochronie roślin jest kontrola chwastów, której znaczenie systematycznie narasta. Poszukiwanie nowych, bezpieczniejszych rozwiązań w tym obszarze stało się więc jednym z priorytetów współczesnej ochrony roślin [10]. Idealnym rozwiązaniem byłyby związki gatunkowo selektywne, biodegradowalne, nietoksyczne dla organizmów niecelowych, a zarazem skuteczne w zwalczaniu chwastów i zdolne do przełamywania ich odporności na herbicydy [7]. Szczególną uwagę w tym kontekście przyciągają produkty wtórnego

metabolizmu roślin, spośród których interesującą grupę stanowią olejki eteryczne (ang. *essential oils*, EO) [6,10].

EO występują w różnych organach roślin (kwiatkach, liściach, kłęczach, nasionach, owocach, drewnie i korze) i są gromadzone w wyspecjalizowanych strukturach wydzielniczych, takich jak pojedyncze komórki gruczołowe, zbiorniki i kanały wydzielnicze oraz włoski gruczołowe (trichomy). Nadają one roślinom charakterystyczny zapach i smak, a ponadto pełnią funkcje związków semiochemicznych – przyciągają zapylacze, odstraszają szkodniki, wykazują właściwości allelopatyczne wobec fitopatogenów, mikroorganizmów glebowych i innych roślin, a także uczestniczą w komunikacji międzysobicznej oraz adaptacji do stresu abiotycznego i biotycznego [6]. EO składają się zwykle z 20–60 aktywnych związków chemicznych, których skład ilościowy i jakościowy zależy od gatunku, poziomu ploidalności, warunków klimatycznych, a także obecności endofitów. Dotychczas zidentyfikowano około 3000 olejków eterycznych. Substancje czynne, które w nich występują, klasyfikuje się do terpenów (monoterpeny, seskwiterpeny), pochodnych węglowodorów (alkohole, fenole, aldehydy, estry) oraz fenylopropanoidów (eugenol, anetol, safrol) [6,11]. W mieszaninach zwykle dominuje od jednej do kilku substancji uznawanych za główne nośniki aktywności biologicznej, natomiast związki występujące w mniejszych ilościach modulują ich efekt, działając synergistycznie [12,13].

EO wykazują szerokie spektrum aktywności biologicznej – przeciwutleniającej, przeciwzapalnej i antyseptycznej. Hamują rozwój bakterii patogennych, w tym szczepów opornych na antybiotyki, a także grzybów i wirusów, co przyczyniło się do ich szerokiego zastosowania w przemyśle spożywczym, kosmetycznym, farmaceutycznym i medycznym [14]. Postęp badań w tych obszarach umożliwił zgromadzenie zaawansowanej wiedzy na temat oddziaływania EO na komórki zwierzęce i ludzkie, w tym nowotworowe [15–18]. Wykazano istotną rolę EO i ich głównych składników w modulacji ekspresji genów, hamowaniu polimeraz DNA odpowiedzialnych za replikację i naprawę DNA oraz w ochronie materiału genetycznego przed uszkodzeniami oksydacyjnymi [19].

Znacznie słabiej rozwinięty pozostaje natomiast obszar badań nad praktycznym wykorzystaniem olejków eterycznych w rolnictwie. Pomimo licznych doniesień, z lat 70. – 90. XX wieku, dotyczących zróżnicowanego wpływu EO na kiełkowanie nasion i wzrost siewek wielu gatunków roślin, postęp w tej dziedzinie był ograniczony w porównaniu z badaniami biomedycznymi. Dostępne prace podkreślały bioherbicydowy potencjał olejków, lecz koncentrowały się głównie na obserwacji efektów fenotypowych, rzadko wyjaśniając molekularne mechanizmy działania poszczególnych składników czy reakcje roślin na poziomie genomu, epigenomu, transkryptomu, proteomu, metabolomu czy ultrastruktury komórkowej. Ograniczenia te wynikały zarówno z mniejszej dostępności technik molekularnych w badaniach materiału roślinnego, jak i ze zbieżności tych prac z okresem intensywnej chemizacji rolnictwa, kiedy wprowadzano na rynek nowe, skuteczne, syntetyczne środki ochrony roślin. Brak zainteresowania naturalnymi preparatami ze strony przemysłu rolnego, niedostatek regulacji prawnych w Europie oraz marginalizowanie problemu degradacji środowiska dodatkowo spowolniły rozwój tego kierunku badań [7,20]. Dopiero obecnie, wraz ze wzrostem świadomości ekologicznej i pojawieniem się alarmujących danych o skutkach stosowania herbicydów chemicznych, obserwuje się intensyfikację prac nad molekularnym podłożem fitotoksyczności substancji pochodzenia naturalnego, w tym olejków eterycznych.

Ze względu na ogromną różnorodność olejków eterycznych oraz ich zmienny skład chemiczny – zależny od gatunku i siedliska rośliny, wrażliwości na czynniki środowiskowe, lotnego charakteru związków i ich ograniczonej rozpuszczalności w wodzie [6,21] – usystematyzowanie dostępnej wiedzy oraz określenie molekularnych mechanizmów działania EO stanowi jedno z głównych wyzwań badawczych w tej

dziedzinie. Dodatkową trudność stwarza fakt, że reakcje roślin mogą różnić się nie tylko między rodzinami i gatunkami, lecz także w zależności od wrażliwości poszczególnych tkanek, zastosowanej dawki, sposobu aplikacji oraz warunków środowiskowych [7].

Dotychczasowe badania nad oddziaływaniem olejków eterycznych (EO) na rośliny dostarczyły już wielu cennych informacji. Ze względu na to, że EO zbudowane są z małych hydrofobowych cząsteczek [6], mogą łatwo przenikać przez sieć ściany komórkowej i oddziaływać bezpośrednio na plazmalemę [22]. Prowadzi to do modyfikacji organizacji lipidowej błon, obejmujących zmiany ich płynności, składu fosfolipidów oraz aktywności enzymów błonowych, co pociąga za sobą istotne konsekwencje komórkowe [23]. Dodatkowo EO indukują powstawanie reaktywnych form tlenu (ang. *reactive oxygen species*, ROS), takich jak rodniki ponadtlenkowe, rodniki hydroksylowe czy nadtlenek wodoru [24]. ROS nasilają peroksydację lipidów i pogłębiają utratę integralności błon, czego objawem jest m.in. wyciek elektrolitów i wzrost przewodnictwa elektrycznego roztworu [25,26].

Destabilizacja błon biologicznych zakłóca funkcjonowanie kluczowych organelli, a tym samym procesów decydujących o metabolizmie rośliny. W chloroplastach prowadzi do spadku zawartości chlorofilu i innych barwników fotosyntetycznych, zaburzeń w transporcie elektronów oraz obniżenia efektywności fotosyntezy i produkcji ATP [27]. Podobne skutki obserwuje się w mitochondriach, gdzie uszkodzenia błon ograniczają oddychanie komórkowe i fosforylację oksydacyjną [28]. Konsekwencją takich zaburzeń jest także osłabienie proliferacji komórek merystematycznych, warunkującej wzrost korzeni, pędów i siewek. Mechanizm ten wiąże się zarówno z zakłóceniami w syntezie DNA jądrowego i organellowego, jak i destabilizacją cytoszkieletu, problemami w formowaniu mikrotubul oraz nieprawidłowym przebiegiem mitozy [29–32].

W literaturze podkreśla się, że mimo licznych danych dotyczących efektów fenotypowych i biochemicznych, wciąż brakuje badań nad wpływem EO na ekspresję genów, metabolizm i szlaki sygnalizacyjne roślin [11,22,33]. Szczegółowe powiązania molekularne wymagają więc dalszych analiz, zwłaszcza że wiele olejków pozostaje pod tym względem całkowicie nieprzebadanych.

Pomimo że olejki eteryczne mogą oddziaływać na komórki roślinne na wielu poziomach, od błon komórkowych po procesy metaboliczne, ich stosowanie charakteryzuje się wysokim bezpieczeństwem dla środowiska i organizmów niedocelowych [1,6]. Olejki eteryczne charakteryzują się wysoką podatnością na degradację – są wrażliwe na promieniowanie świetlne, zmiany temperatury i łatwo ulegają rozkładowi w wyniku oksydacji, izo- i polimeryzacji czy dehydrogenacji [1,6]. Dzięki temu nie stanowią trwałego zagrożenia dla środowiska. Co więcej, ich ostra toksyczność jest stosunkowo niska i ujawnia się dopiero przy dawkach przekraczających 2 g/kg masy ciała w przypadku podania doustnego lub skórniego [6]. Dodatkowym argumentem przemawiającym za bezpieczeństwem stosowania EO jest fakt, że ich pozostałości w tkankach roślinnych są minimalne, co odróżnia je od wielu syntetycznych środków ochrony roślin.

Jednym z EO wykazujących szczególnie interesujące właściwości jest olejek tatarakowy (SEO) pozyskiwany z kłączy tataraku zwyczajnego (*Acorus calamus* L.) rośliny używanej w tradycyjnej medycynie chińskiej [34–36]. Skład chemiczny tego olejku jest zróżnicowany i obejmuje monoterpeny, seskwiterpeny oraz fenylopropanoidy, spośród których szczególną rolę odgrywają azarony: α -azaron, β -azaron (uważany za najbardziej toksyczny) oraz γ -azaron [21,37,38]. Proporcje poszczególnych azaronów w mieszaninie olejku determinuje stopień ploidalności tataraku. Diploidalne odmiany ($2n = 2x = 24$), obecne głównie w Ameryce Północnej i na Syberii, zawierają jedynie 2% olejku, z minimalnym udziałem β -azaronu. Odmiany triploidalne ($2n = 3x = 36$), występujące w Europie i umiarkowanych

rejonach Azji, wytwarzają 3% olejku, z zawartością β -azaronu na poziomie 5–19%. Najczęściej wykorzystywane przemysłowo są jednak odmiany tetraploidalne ($2n = 4x = 48$), charakterystyczne dla wschodniej Azji, Japonii i Indii. Produkują one do 6% olejku, w którym β -azaron stanowi aż 70–96% całkowitej zawartości [21,37].

Badania wskazują, że działanie azaronów może być zarówno korzystne, jak i szkodliwe, w zależności od modelu badawczego i stosowanego stężenia. Wykazują one m.in. właściwości neuroprotektoryjne, przeciwdepresyjne, nasenne, przeciwłękowe, przeciwastmatyczne, hipolipemiczne i przeciwnowotworowe [35,36,39,40]. Z drugiej strony, doniesienia naukowe potwierdzają także ich potencjalne działanie kardiotoksyczne, hepatotoksyczne, reprotoksyczne, mutagenne, a nawet rakotwórcze [21,41].

Na poziomie komórkowym azaron wykazuje działanie dwutorowe antyoksydacyjne lub prooksydacyjne, zależne od stężenia. W niskich stężeniach (0,01–0,1%) działa protekcyjnie, neutralizując wolne rodniki tlenowe, ograniczając peroksydację lipidów oraz aktywując szlaki sygnałowe prowadzące do wzrostu ekspresji enzymów antyoksydacyjnych. W stężeniach wysokich (>0,5%) indukuje jednak powstawanie reaktywnych form tlenu, nasilając peroksydację lipidów, co prowadzi do uszkodzeń błon komórkowych i utraty ich funkcji [18].

W literaturze dostępnych jest niewiele badań dotyczących odpowiedzi roślin na olejek tatarakowy czy azarony. Dotychczas koncentrowały się one głównie na analizie wpływu na kiełkowanie nasion i wczesny rozwój siewek. Fitotoksyczność olejku została potwierdzona m.in. dla sałaty (*Lactuca sativa* L.), życicy trwałej (*Lolium perenne* L.) [41] oraz korzeni zarodkowych cebuli (*Allium cepa* L.), w odniesieniu do progresji podziałów mitotycznych [42].

Pomimo potwierdzonej fitotoksyczności SEO w nielicznych gatunkach roślin, brak jest szerokich badań potwierdzających jego działanie oraz szczegółowych analiz na poziomie fizjologicznym, cytologicznym i molekularnym. Zrozumienie mechanizmów jego wpływu na podstawowy proces jakim jest proliferacja komórek roślinnych, towarzyszący roślinie przez cały cykl życia od kiełkowania po wytwarzanie nasion, jest niezbędne zarówno dla rozwinięcia potencjalnych zastosowań SEO jako naturalnego bioherbicydu, jak i dla oceny jego bezpieczeństwa oraz skuteczności w kontekście rolniczym [43,44]. Choć bioherbicydy, uznawane są za obiecującą alternatywę dla środków syntetycznych, ich wdrożenie w praktyce rolniczej napotyka liczne trudności. Wiele substancji nie spełnia wymogów selektywności lub wykazuje zbyt wąskie spektrum działania, a nawet te o wysokiej skuteczności muszą pokonać długie i złożone procedury rejestracyjne, przez co upływa wiele lat, zanim nowe środki trafią do powszechnego użytku [20]. Dlatego już na etapie badań podstawowych istotne jest systematyczne badanie potencjału naturalnych metabolitów jako przyszłych środków ochrony roślin. W tym kontekście olejki eteryczne, wyróżniające się złożonym składem i wielokierunkowym działaniem, wykazują potencjał zarówno do skutecznego ograniczania chwastów, jak i do zmniejszania ryzyka nabywania przez nie oporności. Dalsze badania obejmujące różne gatunki i rodziny roślin pozwolą lepiej zrozumieć zakres działania SEO i ocenić jego zastosowanie w szerszym kontekście rolniczym.

6.2. Cel pracy i hipotezy badawcze

Celem przeprowadzonych badań była ocena potencjału fitotoksycznego olejku tatarakowego (SEO), ustalenie selektywności jego działania w stosunku do wybranych roślin, a także analiza zmian jakie wywołuje na poziomie struktury komórki, jej metabolizmu, aparatu genetycznego i sieci sygnałnych.

Badania zostały podzielone na 5 etapów, z których każdy obejmował cele cząstkowe wsparte hipotezą badawczą.

6.2.1. Praca 1 (Załącznik nr 1)

Cel: Określenie składu chemicznego komercyjnie dostępnego olejku eterycznego z kłączy *Acorus calamus* (SEO) przy użyciu wysokosprawnej chromatografii cieczowej sprzężonej z tandemową spektrometrią mas typu kwadrupol-czas przelotu z jonizacją ESI (HPLC-ESI-QTOF-MS/MS).

Hipoteza: Głównymi składnikami SEO są fenylopropanoidy, a w szczególności azarony (α -, β - i γ -azaron)

Cele etapowe:

1. Analiza składu SEO przy pomocy HPLC-ESI-QTOF-MS/MS.
2. Oznaczenia zawartości β -azaronu w komercyjnie dostępnym SEO.

6.2.2. Praca 2 (Załącznik nr 2)

Cel: Ocena fitotoksyczności SEO wobec siewek z rodzin Fabaceae i Brassicaceae, obejmująca wyznaczenie wartości IC_{50} dla wzrostu korzeni zarodkowych po 24-godzinnej ekspozycji oraz analizę fizjologicznych i metabolicznych efektów działania olejku.

Hipoteza: SEO przejawia selektywny potencjał bioherbicydowy, ograniczając wzrost siewek z rodziny Brassicaceae przy jednoczesnym zachowaniu tolerancji roślin Fabaceae, co czyni go potencjalnym narzędziem w rolnictwie selektywnym.

Cele etapowe:

1. Wyznaczenie wartości IC_{50} dla wzrostu korzeni zarodkowych siewek *Fabaceae* i *Brassicaceae* po 24-godzinnej ekspozycji na SEO.
2. Analiza efektów fizjologicznych działania SEO, obejmująca: nawodnienie tkanek, syntezę biomasy (świeżej i suchej masy), wyciek elektrolitów do pożywki oraz żywotność komórek.
3. Ocena akumulacji reaktywnych form tlenu (H_2O_2) w korzeniach zarodkowych.
4. Charakterystyka nieenzymatycznego systemu antyoksydacyjnego poprzez oznaczenie poziomu askorbinianu, glutationu, cysteiny, γ -glutamylcysteiny oraz α - i β -karotenu.
5. Ocena aktywności enzymatycznych mechanizmów antyoksydacyjnych: SOD, APX, GR i CAT.
6. Analiza ogólnej aktywności metabolicznej siewek metodą kalorymetrii izotermicznej.
7. Badanie wpływu SEO na profil metaboliczny (białka, cukry proste oraz nasycone i nienasycone kwasy tłuszczowe).
8. Ocena uszkodzeń oksydacyjnych w komórkach na podstawie poziomu peroksydacji lipidów (MDA).

6.2.3. Praca 3 (Załącznik nr 3)

Cel: Analiza wpływu SEO na interfazę komórek proliferujących w merystemie korzeni zarodkowych po 24-godzinnej ekspozycji, ze szczególnym uwzględnieniem dynamiki replikacji DNA i integralności materiału genetycznego.

Hipoteza: SEO hamuje wzrost korzeni zarodkowych poprzez indukcję stresu oksydacyjnego, co prowadzi do zaburzeń procesu replikacji DNA w fazie S. Zaburzenia te mogą być ponadto specyficzne dla gatunku lub różnić się pomiędzy rodzinami Fabaceae i Brassicaceae.

Cele etapowe:

1. Ocena stresu oksydacyjnego w komórkach merystematycznych poprzez wizualizację reaktywnych form tlenu (ROS), w tym H_2O_2 (DAB) i O_2^- (NBT).
2. Określenie procentowego udziału komórek w poszczególnych etapach interfazy cyklu komórkowego (G1, S, G2) za pomocą cytofotometrycznego pomiaru zawartości DNA.
3. Analiza przebiegu replikacji DNA w fazie S poprzez oznaczenie procentowego udziału komórek inkorporujących EdU oraz ocenę dynamiki włączania EdU i wzorów znakowania fluorescencyjnego charakterystycznych dla początkowego, zaawansowanego i końcowego etapu fazy S.
4. Ocena uszkodzeń ciągłości DNA i zdolności komórek do naprawy poprzez oznaczenie γ -H2AX.
5. Weryfikacja degradacji DNA na poziomie komórkowym z zastosowaniem testu TUNEL.
6. Całościowa ocena degradacji DNA w merystemach korzeni przy użyciu elektroforezy w żelu agarozowym.
7. Porównanie odpowiedzi między gatunkami i rodzinami w kontekście obserwowanych zakłóceń w replikacji DNA i integralności materiału genetycznego.

6.2.4. Praca 4 (Załącznik nr 4)

Cel: Analiza wpływu SEO na przebieg mitozy w komórkach merystematycznych z korzeni zarodkowych po 24-godzinnej ekspozycji, ze szczególnym uwzględnieniem regulatorów cyklu komórkowego, organizacji cytoszkieletu oraz specyficznych dla mitozy modyfikacji epigenetycznych.

Hipoteza: SEO hamuje wzrost korzeni zarodkowych poprzez zaburzenia mitozy wynikające z dysfunkcji molekularnych mechanizmów regulacyjnych, zakłóceń w organizacji wrzeciona mitotycznego oraz remodelingu chromatyny. Charakter i intensywność tych zaburzeń mogą być ponadto specyficzne dla gatunku lub różnicować się pomiędzy rodzinami Fabaceae i Brassicaceae.

Cele etapowe:

1. Ocena poziomu głównej kinazy regulatorowej Cdc2 w komórkach merystematycznych z korzeni zarodkowych.
2. Wyznaczenie procentowego udziału komórek w mitozie i jej kolejnych etapach oraz identyfikacja i ilościowa analiza aberracji chromosomowych.
3. Analiza organizacji i remodelingu wrzeciona mitotycznego oraz włókien aktynowych w komórkach mitotycznych.
4. Ocena specyficznych dla mitozy modyfikacji epigenetycznych histonu H3 polegających na fosforylacji treoniny 3 i seryny 10 (H3T3Ph i H3S10Ph), odpowiedzialnych za prawidłową segregację chromatyd.

5. Porównanie obserwowanych efektów między gatunkami i rodzinami w kontekście obserwowanych zakłóceń w przebiegu mitozy, organizacji wrzeciona i modyfikacji epigenetycznych.

6.2.5. Praca 5 (Załącznik nr 5)

Cel: Analiza wpływu SEO na aktywność merystemu wierzchołkowego korzeni zarodkowych poprzez ocenę sygnalizacji metabolicznej zależnej od cukru i kaskad MAPK, a także udziału tych sygnałów w przeprogramowaniu epigenetycznym chromatyny w kontekście modyfikacji profilu transkrypcyjnego oraz reorganizacji strukturalnych związanych z naprawą DNA.

Hipoteza: SEO hamuje wzrost korzeni zarodkowych poprzez indukcję stresu, który zaburza sygnalizację metaboliczną i aktywuje kaskady MAPK. Efektem tych procesów jest epigenetyczna rekonfiguracja chromatyny, chroniąca stabilność genomu poprzez ograniczenie ogólnej aktywności transkrypcyjnej przy jednoczesnej selektywnej aktywacji genów kluczowych dla adaptacji i przeżycia komórek. Charakter i intensywność tych modyfikacji mogą być ponadto specyficzne dla gatunku lub różnicować się pomiędzy rodzinami Fabaceae i Brassicaceae.

Cele etapowe:

1. Analiza aktywności heksokinazy (HXK) jako głównego enzymu w sygnalizacji cukrowej.
2. Ocena akumulacji ufosforylowanych form MAPK w komórkach merystematycznych.
3. Określenie globalnych poziomów metylacji DNA w komórkach merystematycznych.
4. Analiza modyfikacji histonów związanych z reorganizacją chromatyny, w tym H3K4Me2, H3T45Ph, H4K5Ac, H3K9Ac i H3K56Ac pod kątem represji, aktywacji genów oraz odpowiedzi na uszkodzenia DNA.
5. Ocena aktywności transkrypcyjnej poprzez znakowanie nowo powstającego RNA testem EU.
6. Analizy ekspresji wybranych genów (HXK, SuSy, MKK) związanych z odpowiedzią na stres metodą qPCR.
7. Analiza morfologii i aktywności jąderka za pomocą barwienia AgNOR.
8. Charakterystyka ultrastrukturalnych zmian w komórkach merystematycznych z zastosowaniem transmisyjnej mikroskopii elektronowej.
9. Porównanie obserwowanych efektów między gatunkami i rodzinami w kontekście obserwowanych zakłóceń w sygnalizacji, remodelingu chromatyny, aktywności transkrypcyjnej oraz ultrastruktury komórki.

6.3. Metodyka badań

6.3.1. Model badawczy

6.3.1.1. Materiał badawczy

Model badawczy obejmował młode (2-3 dniowe) siewki czterech gatunków roślin należących do dwóch rodzin, wybranych ze względu na ich rolę w rolnictwie oraz znaczenie w badaniach naukowych. Do analizy włączono dwa gatunki uprawne z rodziny Fabaceae: bobik (*Vicia faba* L. var. *minor*) i łubin żółty (*Lupinus luteus* L.) oraz dwa gatunki chwastów z rodziny Brassicaceae: rzepak ozimy (*Brassica napus* L.) i rzodkiewnik pospolity (*Arabidopsis thaliana* L.).

Uwzględnienie roślin z rodziny Fabaceae wynikało z ich dużego potencjału produkcyjnego oraz trudności związanych z ochroną upraw przed konkurencją chwastów. Choć areał upraw Fabaceae w Polsce jest ograniczony ze względu na wrażliwość tych roślin na zmienne warunki pogodowe i suszę, to według raportu Organizacji Narodów Zjednoczonych do spraw Wyżywienia i Rolnictwa (ang. *Food and Agriculture Organization*, FAO) kraj zajmuje drugie miejsce na świecie pod względem produkcji łubinu — w tym łubinu żółtego, białego i wąskolistnego. Rośliny te dostarczają nasion o dużej masie i wysokiej zawartości białka, wykorzystywanych przede wszystkim jako pasza w żywieniu zwierząt. Jednocześnie pełnią funkcję międzyplonów, wzbogacających glebę w materię organiczną i azot, które sprzyjają poprawie jej żyzności. Istotnym problemem pozostaje jednak powolny początkowy wzrost siewek i ograniczona dostępność herbicydów (obecnie zarejestrowano dziewięć substancji czynnych w przypadku bobiku i siedem w przypadku łubinu), co sprzyja presji chwastów dwuliściennych. Szczególne zagrożenie stanowi *B. napus*, który mimo dużego znaczenia gospodarczego, w postaci samosiewów utrudnia prowadzenie upraw strączkowych, zwłaszcza w systemach monokulturowych [45]. Z kolei *A. thaliana* pełni podwójną rolę, jest zarówno chwastem, jak i uznanym organizmem modelowym. Dzięki całkowicie zsekwencjonowanemu genomowi i powszechnemu zastosowaniu w badaniach molekularnych stanowi istotny punkt odniesienia oraz cenny obiekt w dalszych analizach. Na uwagę zasługują także inne cechy badanych gatunków, istotne z punktu widzenia metodologii. *V. faba* var. *minor* jest doskonałym materiałem do badań cytologicznych dzięki dużym i wyraźnie widocznym w analizach mikroskopowych chromosomom. Z kolei *L. luteus* i *B. napus*, charakteryzujące się niską zawartością DNA i siateczkowatą strukturą chromatyny interfazowej, szczególnie dobrze nadają się do porównawczych immunofluorescencyjnych analiz epigenetycznych. Dobór takiego zestawu gatunków umożliwił stworzenie spójnego systemu modelowego do analizy mechanizmów działania SEO w tkankach korzeni zarodkowych młodych siewek. W odróżnieniu od wcześniejszych prac, koncentrujących się głównie na kiełkowaniu nasion lub hamowaniu mitozy [41,42], przeprowadzone analizy objęły różne poziomy organizacji (od strukturalnego i fizjologicznego po cytologiczny i molekularny) przy uwzględnieniu gatunków reprezentujących dwie istotne dla rolnictwa rodziny. Takie podejście pozwoliło na uzyskanie szerszej, bardziej mechanistycznej perspektywy w ocenie reakcji roślin na naturalne allelochemikalia.

6.3.1.2. Olejek eteryczny z tataraku zwyczajnego (SEO)

Wybór olejku eterycznego z tataraku zwyczajnego (*Acorus calamus* L.) do niniejszych badań wynikał z jego unikatowego składu chemicznego, a w szczególności z obecności fenylopropanoidów (takich jak azarony [38,41]), znanych z właściwości modulujących procesy redoks. Pomimo wielowiekowego zastosowania w medycynie tradycyjnej oraz udokumentowanej aktywności biologicznej w modelach zwierzęcych i systemach *in vitro* [17,18,42] mechanizmy molekularne działania tego olejku w

komórkach roślinnych pozostają słabo poznane. O wyborze SEO zdecydowały również względy praktyczne polegające na opłacalności ekonomicznej wynikającej z łatwej dostępności surowca oraz dużych kłaczy stanowiących bogate źródło olejku.

Ze względu na hydrofobowy charakter olejków eterycznych, SEO emulgowano w celu zwiększenia stabilności, biodostępności i równomierności działania [6,11]. Olejek rozprowadzono w mieszaninie złożonej z nasyconych kwasów tłuszczowych C14–18, nienasyconych C16–18 mono- i di-etoksyloowanych glicerydów oraz etoksylowanego oleju *B. napus* w proporcji 1:4 (v/v), zgodnie z protokołem stosowanym w badaniach szklarniowych [46]. Proporcję tę przyjęto na podstawie wcześniejszych analiz, które potwierdziły skuteczność emulsji. Forma niezemulgowana powodowała nierównomierny kontakt SEO z korzeniami i dużą zmienność wyników, dlatego w badaniach wykorzystano wyłącznie postać zemulgowaną.

6.3.1.3. Hodowle roślin

Dla oceny wpływu SEO na pierwotny wzrost korzeni zarodkowych przygotowano roztwory w zakresie stężeń 0,01–0,05% dla *V. faba*, *L. luteus* i *B. napus* oraz 0,004–0,009% dla *A. thaliana*, u którego wyższe dawki całkowicie blokowały wzrost. Siewki inkubowano przez 24 godziny w szczelnie zamkniętych szalkach Petriego. Kontrole obejmowały hodowlę w wodzie oraz w roztworach emulgatora w stężeniach użytych do emulgowania olejku. Na tej podstawie wyznaczono wartości IC_{50} (dawki hamujące wzrost korzeni o 50%) dla każdego gatunku, przyjmując przyrost długości korzenia jako parametr odzwierciedlający zarówno proliferację komórek merystematycznych, jak i ich ekspansję w strefie wydłużeniowej. Określenie wartości IC_{50} umożliwiło ocenę potencjału fitotoksycznego SEO, porównanie reakcji przedstawicieli różnych grup taksonomicznych, identyfikację wzorców wrażliwości charakterystycznych dla poszczególnych gatunków i rodzin oraz analizę potencjalnych celów komórkowych olejku przy równoważnym natężeniu stresu. Wyznaczone w ten sposób wartości IC_{50} (0,03 % dla *V. faba*, 0,025 % dla *L. luteus*, 0,01 % dla *B. napus* oraz 0,005 % dla *A. thaliana*) były priorytetowe i stały się podstawą wszystkich kolejnych eksperymentów, w których hodowla odbywała się przez 24 godziny.

6.3.2. Metody badawcze

(Praca 1; Załącznik nr 1)

6.3.2.1. Metody chromatograficzne i spektroskopowe

6.3.2.1.1. Analiza składu chemicznego olejku eterycznego

Skład olejku eterycznego pozyskanego z kłaczy tataraku zwyczajnego (*Acorus calamus* L.) określono z wykorzystaniem wysokosprawnej chromatografii cieczowej sprzężonej z tandemową spektrometrią mas (HPLC-ESI-QTOF-MS/MS). Zastosowana technika łączyła rozdział mieszaniny w kolumnie chromatograficznej z identyfikacją uzyskanych związków przy użyciu spektrometru mas. Faza ruchoma, stanowiąca mieszaninę rozpuszczalników, transportowała próbkę przez kolumnę chromatograficzną, w której poszczególne składniki olejku ulegały separacji w zależności od powinowactwa do fazy stacjonarnej. Następnie eluujące się związki poddawano jonizacji w źródle ESI, w którym pod wpływem wysokiego napięcia (ok. 1 kV) powstawały jony molekularne. Uzyskane jony wprowadzano do tandemowego układu analizatorów QTOF. W pierwszym analizatorze selekcjonowano jony macierzyste o określonym stosunku masy do ładunku (m/z), które następnie kierowano do komory fragmentacji, gdzie dochodziło do ich rozpadu na jony potomne. Uzyskane widma masowe porównywano z danymi referencyjnymi w dostępnych bazach danych, co umożliwiło identyfikację poszczególnych komponentów chemicznych obecnych w badanym oleju.

6.3.2.2. Metody morfometryczne

6.3.2.2.1. Ocena przyrostu korzeni i wyznaczenie wartości IC_{50}

Metoda morfometryczna umożliwiła ocenę wpływu SEO na wczesny wzrost siewek poprzez pomiar długości korzeni zarodkowych. Podstawowym celem analiz było wyznaczenie wartości IC_{50} , czyli stężenia SEO hamującego wzrost korzeni o 50%, co pozwoliło ustalić porównywalny poziom stresu dla wszystkich gatunków, stosowany we wszystkich kolejnych badaniach fizjologicznych, cytologicznych i molekularnych.

6.3.2.2.2. Ocena świeżej i suchej masy korzeni

Pomiary świeżej i suchej masy korzeni umożliwiły określenie, czy spadek biomasy wynikał głównie z ograniczenia syntezy składników strukturalnych, czy z zaburzeń gospodarki wodnej. Wyniki te dostarczyły podstawowych informacji o fizjologii i dynamice wzrostu, uzupełniając analizę przyrostu długości korzeni.

6.3.2.3. Test żywotności komórek

6.3.2.3.1. Spektrofotometryczna ocena żywotności z wykorzystaniem błękitu Evansa

Błękit Evansa nie przenika przez błony plazmatyczne żywych komórek, a gromadzi się wyłącznie w cytoplazmie komórek obumarłych lub o naruszonej integralności błony. Połączenie barwienia z analizą spektrofotometryczną umożliwia ilościową ocenę stopnia uszkodzeń i porównanie reakcji w różnych wariantach doświadczenia.

6.3.2.4. Test integralności błon komórkowych

6.3.2.4.1. Analiza konduktywności medium hodowlanego

Uszkodzenia błon komórkowych powodują wyciek jonów zwiększając przewodność medium hodowlanego. Pomiar konduktywności umożliwił szybkie i ilościowe określenie stopnia uszkodzeń błon w reakcji na SEO, stanowiąc uzupełnienie testu żywotności.

6.3.2.5. Ocena stresu oksydacyjnego i systemu antyoksydacyjnego

6.3.2.5.1. Spektrofotometryczna analiza zawartości nadtlenu wodoru (H_2O_2)

Pmiar zawartości H_2O_2 stanowi podstawowy wskaźnik stresu oksydacyjnego w komórkach roślinnych, ponieważ H_2O_2 jest trwałą i łatwą do oznaczenia formą reaktywnych form tlenu, której akumulacja odzwierciedla nasilenie stresu oksydacyjnego w tkankach. W przeciwieństwie do bardzo reaktywnych i krótkotrwałych ROS, takich jak anionorodnik ponadtlenkowy ($O_2^{\cdot-}$) czy rodnik hydroksylowy ($\cdot OH$), H_2O_2 jest wystarczająco stabilny, by umożliwić wiarygodne oznaczenia ilościowe. Parametr ten stanowił podstawę do porównań międzygatunkowych a w powiązaniu z parametrami, takimi jak peroksydacja lipidów czy aktywność enzymów antyoksydacyjnych umożliwił interpretację mechanizmów obronnych wobec SEO.

6.3.2.5.2. Spektrofotometryczna analiza aktywności enzymów antyoksydacyjnych (SOD, CAT, APX, GR) oraz askorbinianu

Ocena aktywności enzymów antyoksydacyjnych, takich jak dysmutaza ponadtlenkowa (SOD), katalaza (CAT), peroksydaza askorbinianowa (APX) oraz reduktaza glutationowa (GR), oraz zawartości askorbinianu, pozwoliła określić zdolność roślin do detoksykacji reaktywnych form tlenu i utrzymania

równowagi redoks w komórkach. Zestawienie danych enzymatycznych i nieenzymatycznych umożliwiło całościową charakterystykę systemu antyoksydacyjnego.

6.3.2.5.3. Analiza zawartości glutationu, cysteiny, γ -glutamylcysteiny i karotenoidów z użyciem HPLC

Nieenzymatyczne przeciwutleniacze (glutationu, cysteina, γ -glutamylcysteina) w fazie wodnej komórki współdziałają z enzymami antyoksydacyjnymi, zapewniając skuteczną obronę przed stresem oksydacyjnym. Karotenoidy, rozpuszczalne w lipidach, pełnią natomiast rolę antyoksydantów w błonach, chroniąc lipidy przed uszkodzeniami oksydacyjnymi i stabilizując strukturę fazy lipidowej komórek. Wysokosprawna chromatografia cieczowa (HPLC) umożliwia jednocześnie rozdzielenie i ilościowe oznaczenie tych związków w złożonych próbkach tkankowych. Metoda zapewnia wysoką czułość, selektywność i powtarzalność, pozwalając na dokładne porównywanie profili nieenzymatycznych antyoksydantów między gatunkami oraz ocenę ich zmian pod wpływem SEO.

6.3.2.5.4. Spektrofotometryczna analiza peroksydacji lipidów

Pomiar zawartości malonyldialdehydu (MDA) stanowi ilościowy wskaźnik stopnia peroksydacji lipidów, czyli procesu niszczenia nienasyconych kwasów tłuszczowych w błonach komórkowych pod wpływem reaktywnych form tlenu. MDA powstaje w trakcie tego procesu jako produkt uboczny i w praktyce działa jak „czujnik” uszkodzeń błon, pozwalając ocenić intensywność stresu oksydacyjnego w tkankach. Parametr ten stanowi uzupełnienie danych o aktywności enzymatycznej i poziomie nieenzymatycznych antyoksydantów, pozwalając ocenić, na ile mechanizmy obronne skutecznie chronią błony komórkowe przed uszkodzeniami wywołanymi przez SEO.

6.3.2.6. Ocena aktywności metabolicznej

6.3.2.6.1. Kalorymetria izotermiczna

Kalorymetria izotermiczna umożliwia bezpośredni pomiar aktywności metabolicznej całych siewek poprzez rejestrację ciepła wydzielanego w trakcie procesów biochemicznych. Ponieważ każda reakcja metaboliczna generuje energię cieplną, metoda ta stanowi syntetyczny wskaźnik kondycji metabolicznej nienaruszonej tkanki. Umożliwia ocenę, czy reakcja roślin na SEO przebiega w sposób zrównoważony, czy też towarzyszy jej nadmierna mobilizacja energii, świadcząca o stresie metabolicznym. Jej zaletą jest nieinwazyjny charakter oraz możliwość monitorowania dynamiki zmian w czasie rzeczywistym, co czyni ją wartościowym uzupełnieniem analiz biochemicznych i morfometrycznych.

6.3.2.6.2. Spektrofotometryczna analiza zawartości białek oraz monosacharydów

Białka i cukry proste pełnią podstawowe funkcje energetyczne i strukturalne, a zmiany ich zawartości odzwierciedlają syntezę biomasy, mobilizację rezerw i adaptację metaboliczną w warunkach stresu. Oznaczenia spektrofotometryczne umożliwiają szybkie i powtarzalne określenie poziomu tych związków przy niewielkiej ilości materiału, co pozwala porównywać strategie metaboliczne między gatunkami i wariantami eksperymentalnymi.

6.3.2.6.3. Analiza profilu kwasów tłuszczowych metodą GC/MS.

Profil kwasów tłuszczowych nasyconych (ang. *saturated fatty acids*, SFA), jednonienasyconych (ang. *monounsaturated fatty acids*, MUFA) oraz wielonienasyconych (ang. *polyunsaturated fatty acids*, PUFA) odzwierciedla stan błon komórkowych oraz rezerw lipidowych, a jego zmiany pod wpływem stresu dostarczają informacji o procesach adaptacyjnych. Kluczowe znaczenie mają PUFA, które zwiększają płynność błon, ale równocześnie podnoszą podatność na peroksydację lipidów. Porównanie udziału

SFA, MUFA i PUFA umożliwia więc ocenę odporności błon na stres oksydacyjny. Chromatografia gazowa sprzężona ze spektrometrią mas (GC/MS) stanowi metodę referencyjną o najwyższej czułości i precyzji, łącząc separację estrów metylowych kwasów tłuszczowych (GC) z ich jednoznaczną identyfikacją (MS). Dzięki temu możliwe było wiarygodne, jakościowe i ilościowe porównanie profili lipidowych między gatunkami oraz powiązanie ich zmian z intensywnością peroksydacji.

(Praca 3; Załącznik nr 3)

6.3.2.7. Metody analizy stresu oksydacyjnego i replikacyjnego w merystemach korzeni

6.3.2.7.1. Wizualizacja stresu oksydacyjnego w komórkach proliferujących

6.3.2.7.1.1. Detekcja nadtlenu wodoru z użyciem 3,3'-diaminobenzyny (DAB)

Jest to klasyczna metoda wizualizacji nadtlenu wodoru (H_2O_2) bezpośrednio w tkankach roślinnych, umożliwiając obserwacje w preparatach mikroskopowych przy użyciu mikroskopii świetlnej. Polega na reakcji DAB z H_2O_2 w obecności peroksydaz, w wyniku której powstaje nierozpuszczalny, brązowy osad w miejscach akumulacji H_2O_2 . Metoda pozwala na szybką lokalizację H_2O_2 w komórkach oraz jakościową i półilościową ocenę stopnia jego nagromadzenia. Uzyskane wyniki odzwierciedlają intensywność stresu oksydacyjnego na poziomie komórkowym i dobrze korelują z danymi ilościowymi. Technika ta stanowi cenne narzędzie do wstępnej identyfikacji komórek poddanych stresowi oksydacyjnemu w warunkach działania SEO i stanowi punkt wyjścia do dalszych, bardziej szczegółowych analiz mechanizmów odpowiedzi komórkowej.

6.3.2.7.1.2. Detekcja anionorodnika ponadtlenkowego barwnikiem NBT

Metoda umożliwia wizualizację anionorodnika ponadtlenkowego ($O_2^{\bullet-}$) bezpośrednio w tkankach roślinnych przy użyciu mikroskopii świetlnej. Polega na redukcji barwnika – błękitu nitrotetrazolowego (ang. *nitro blue tetrazolium*, NBT) przez $O_2^{\bullet-}$ do nierozpuszczalnego, niebieskiego formazanu, który odkłada się w miejscach intensywnej produkcji tego rodniaka. Metoda pozwala na bezpośrednie wskazanie komórek z nagromadzeniem reaktywnych form tlenu, a intensywność wybarwienia odzwierciedla poziom stresu oksydacyjnego. Dzięki temu możliwa jest zarówno ocena jakościowa, jak i półilościowa, a wyniki dobrze korelują z danymi ilościowymi. Zaletą techniki jest szybkie uzyskanie informacji na poziomie komórkowym, co stanowi punkt wyjścia do szczegółowych analiz procesów oksydacyjnych w komórkach narażonych na SEO.

6.3.2.8. Analiza procentowego rozkładu komórek w interfazie

6.3.2.8.1. Cytofotometryczny pomiar zawartości DNA

Metoda opiera się na cytofotometrycznym pomiarze zawartości DNA wybarwionego metodą Feulgena w preparatach mikroskopowych z merystemów korzeniowych, przy wykorzystaniu mikroskopii świetlnej oraz komputerowego systemu do mikrodensytometrii. Pozwala określić procentowy udział komórek w poszczególnych fazach interfazy (G1, S, G2) oraz monitorować zmiany wywołane działaniem czynników fitotoksycznych. Analiza rozkładu procentowego umożliwia ocenę funkcjonowania punktów kontrolnych G1/S i G2/M, wykrycie zaburzeń replikacji DNA, oraz ewentualnego przełączenia komórek na endoreplikację. Metoda dostarcza precyzyjnych danych ilościowych niezbędnych do oceny wpływu czynników stresowych na proliferację i regulację cyklu komórkowego.

6.3.2.9. Ocena replikacji DNA

6.3.2.9.1. Analiza inkorporacji 5-etynylo-2'-deoksyurydyny (EdU)

Analiza inkorporacji 5-etynylo-2'-deoksyurydyny (EdU) przy użyciu mikroskopii konfokalnej jest nowoczesną metodą pozwalającą na identyfikację komórek aktywnie replikujących DNA w fazie S cyklu komórkowego. EdU, będący analogiem tymidyny, wbudowuje się w nowo syntetyzowane nici DNA, a reakcja „click chemistry” umożliwia znakowanie fluorescencyjne. Metoda pozwala określić zarówno liczbę komórek replikujących, a w wyniku pomiarów intensywności sygnałów, również dynamikę replikacji w obrębie chromatyny. Ponadto analiza wzorów sygnalizacji umożliwia określenie proporcji komórek w kolejnych etapach fazy S. Komórki o jednolitym, słabym sygnale odpowiadają wczesnej fazie S, gdy liczba aktywnych replikonów jest niewielka; komórki o silnym, homogenicznym sygnale reprezentują środkową fazę S, charakteryzującą się intensywną replikacją; natomiast sygnały w postaci klastrow lokalizują późno replikowaną heterochromatinę, typową dla końcowego etapu fazy S. Metoda EdU w połączeniu z mikroskopią konfokalną, która umożliwia trójwymiarową wizualizację i selektywne zbieranie sygnału fluorescencyjnego z określonych płaszczyzn preparatu, stanowi precyzyjne narzędzie do badania aktywności replikacyjnej na poziomie subjądrowym.

6.3.2.10. Ocena uszkodzeń DNA

6.3.2.10.1. Immunofluorescencyjna detekcja γ -H2AX

Detekcję bardzo wczesnej odpowiedzi komórki na podwójne pęknięcia nici DNA przeprowadzono poprzez immunofluorescencyjne znakowanie histonu H2AX z ufosforylowaną resztą seryny 139 (γ -H2AX). Metoda pozwala wizualizować „ogniska γ -H2AX”, wskazujące miejsca uszkodzeń DNA oraz inicjowane procesy naprawcze. Ocena sygnału umożliwia zarówno analizę jakościową (rozmieszczenie ognisk w jądrach), jak i ilościową (liczba ognisk na jądro), co pozwala określić stopień genotoksyczności indukowanej przez czynniki fitotoksyczne.

6.3.2.10.2. Test TUNEL

Test TUNEL (ang. *Terminal deoxynucleotidyl transferase dUTP Nick End Labeling*) dostarcza danych jakościowych i ilościowych o rozległości i rozmieszczeniu uszkodzeń DNA w tkankach, uzupełniając analizę wczesnych markerów uszkodzeń, takich jak γ -H2AX. Umożliwia on detekcję pęknięć nici DNA z wolnymi końcami 3'-OH poprzez przyłączanie znakowanych nukleotydów katalizowane przez terminalną transferazę deoksynukleotydową (ang. *Terminal deoxynucleotidyl transferase*, TdT). Fluorescencyjna detekcja i ilościowa analiza sygnału, przy zastosowaniu programu ImageJ, pozwala ocenić stopień degradacji DNA w pojedynczych komórkach, niezależnie od aktywacji mechanizmów naprawczych. Umożliwia tym samym określenie, w jakim zakresie uszkodzenia pozostają nienaprawione w wyniku działania czynników stresowych lub fitotoksycznych uzupełniając analizę wczesnych markerów uszkodzeń, takich jak γ -H2AX.

6.3.2.10.3. Elektroforeza DNA w żelu agarozowym

Integralność genomu w całej populacji komórek oceniano metodą elektroforezy DNA w żelu agarozowym. Fragmentacja DNA ujawnia się w postaci smugi lub dodatkowych prążków odpowiadających mniejszym fragmentom, natomiast nienaruszone DNA tworzy wyraźny prążek w okolicy miejsca startu rozdziału elektroforetycznego. Metoda stanowi uzupełnienie analiz immunocytochemicznych (γ -H2AX) oraz TUNEL, pozwalając określić, czy obserwowane w pojedynczych komórkach uszkodzenia prowadzą do globalnej degradacji DNA.

6.3.2.11. Metody analizy przebiegu mitozy w merystemach korzeni

6.3.2.11.1. Ocena aktywności mitotycznej

Na podstawie preparatów z merystemów korzeniowych barwionych metodą Feulgena (specyficzną dla jądrowego DNA), przeprowadzono analizę aktywności podziałowej komórek poprzez wyznaczenie indeksu mitotycznego (MI), definiowanego jako procent komórek znajdujących się w mitozie w stosunku do całkowitej liczby analizowanych komórek. Określano również indeksy fazowe, odzwierciedlające rozkład komórek w poszczególnych fazach mitozy (profaza, metafaza, anafaza, telofaza), co pozwalało ocenić dynamikę przebiegu podziału. Równolegle analizowano częstość występowania aberracji chromosomowych, takich jak fragmentacje, mostki czy opóźnione chromatydy, będących wskaźnikiem zaburzeń integralności genomu i stabilności chromosomowej. Uzyskane parametry stanowiły podstawę ilościowej oceny wpływu czynnika fitotoksycznego na aktywność proliferacyjną oraz prawidłowość przebiegu mitozy w komórkach merystematycznych.

6.3.2.11.2. Detekcja głównego regulatora cyklu komórkowego kinazy Cdc2

Detekcję poziomu kinazy Cdc2 przeprowadzono z wykorzystaniem techniki Western blot. Metoda ta, po elektroforetycznym rozdzieleniu całkowitego ekstraktu białkowego z merystemów w żelu poliakrylamidowym i przeniesieniu na membrany PVDF, umożliwia identyfikację poszukiwanego białka z użyciem przeciwciał swoistych dla kinazy Cdc2 oraz odpowiedniego przeciwciała wtórnego sprzężonego z markerem detekcyjnym. Analiza intensywności prążków z wykorzystaniem programu GelAnalyzer pozwoliła na ilościową ocenę gęstości optycznej sygnału. Cdc2 jest główną kinazą regulatorową przebiegu cyklu komórkowego, utrzymywaną w komórkach na względnie stałym poziomie. Zaobserwowane zmiany w jej zawartości mogą zatem wskazywać na ingerencję czynnika fitotoksycznego w regulację ekspresji i stabilności tego białka.

6.3.2.11.3. Analiza cytoszkieletu

Do analizy organizacji cytoszkieletu zastosowano immunofluorescencję z użyciem przeciwciał swoistych dla tubuliny (mikrotubule) oraz aktyny (mikrofilamenty). Metoda ta pozwala na wizualizację przestrzennego układu głównych elementów cytoszkieletu w komórkach merystematycznych. Fluorescencyjne znakowanie umożliwia ocenę zarówno ciągłości, jak i reorganizacji włókien, co ma istotne znaczenie w badaniach nad odpowiedzią komórek roślinnych na czynniki stresowe i fitotoksyczne. Mikrotubule i mikrofilamenty odgrywają kluczową rolę w kontroli podziałów komórkowych, uczestnicząc w tworzeniu wrzeciona kariokinetycznego i cytokinetycznego, oraz w transporcie wewnątrzkomórkowym. Analiza ich architektury dostarcza zatem ważnych informacji o wpływie stresu na procesy proliferacyjne.

6.3.2.11.4. Analiza modyfikacji epigenetycznych

Do detekcji fosforylacji treoniny 3 i seryny 10 histonu H3 (H3T3Ph i H3S10Ph), ściśle powiązanej z regulacją cyklu komórkowego, zastosowano immunofluorescencję z użyciem swoistych przeciwciał pierwszo- i drugorzędowych sprzężonych z fluorochromem. Metoda ta umożliwia wizualizację w mikroskopii fluorescencyjnej przestrzennego rozkładu wybranych modyfikacji bezpośrednio w chromosomach mitotycznych. Analiza H3T3Ph i H3S10Ph dostarcza informacji nie tylko o zmianach strukturalnych, lecz także o aktywności regulatorowej maszynerii cyklu komórkowego, ponieważ modyfikacje te są dynamicznie nakładane i usuwane przez specyficzne kinazy i fosfatazy w ściśle określonych momentach mitozy. Ilościowa analiza sygnału z wykorzystaniem programu ImageJ pozwala

określić, czy zaburzenia w podziałach komórkowych wynikają głównie z ingerencji w mechanizmy regulacyjne, czy też stanowią konsekwencję zmian w strukturze cytoszkieletu.

(Praca 5; Załącznik nr 5)

6.3.2.12. Metody badania struktury, sygnalizacji i remodelingu chromatyny w regulacji procesów komórkowych w merystemach korzeni

6.3.2.12.1. Analizy sygnalizacji komórkowej

6.3.2.12.1.1. Analizy aktywności heksokinazy (HXK)

HXK pełni podwójną funkcję – jest zarówno kluczowym enzymem inicjującym metabolizm węglowodanów (glikolizę), jak i ważnym białkiem sygnałowym. Jej aktywność odzwierciedla zarówno poziom zaopatrzenia komórek w energię, jak i funkcjonowanie szlaków sygnalizacyjnych związanych z proliferacją i żywotnością komórek. Aktywność HXK w komórkach merystematycznych wykrywano wykorzystując fosforylację heksozy (glukozy) do glukozo-6-fosforanu, który w obecności specyficznej dehydrogenazy i NADP⁺ redukuje NBT do nierozpuszczalnego, barwnego formazanu widocznego w mikroskopii świetlnej. Analiza intensywności zabarwienia z zastosowaniem programu ImageJ, a tym samym aktywności HXK, stanowi cenne narzędzie do oceny wpływu czynników fitotoksycznych na podstawowe procesy metaboliczne i sygnałowe, determinujące prawidłowe funkcjonowanie komórek merystematycznych.

6.3.2.12.1.2. Analiza aktywności kinaz MAP

Aktywność kinaz sygnałowych MAPK (MPK3 i MPK6, funkcjonalnych analogów ERK1/2 u zwierząt, odgrywających istotną rolę w odpowiedzi na stres i regulacji cyklu komórkowego) analizowano metodą immunofluorescencyjną oraz techniką Western blot. Immunofluorescencja umożliwia identyfikację sygnału bezpośrednio w komórkach, pozwalając na ocenę rozmieszczenia aktywnych kinaz i wykazanie translokacji sygnału (lub jej braku) do jądra, co stanowi istotny etap przekazywania informacji do aparatu transkrypcyjnego. Równolegle zastosowano analizę Western blot, która dostarcza ilościowych danych o poziomie ufosforylowanych kinaz w całej populacji komórek. Połączenie obu metod okazało się szczególnie przydatne w badaniach porównawczych: gdyż w komórkach merystematycznych *V. faba* sygnał jądrowy był dobrze widoczny w mikroskopii fluorescencyjnej, podczas gdy w przypadku *B. napus* detekcja jądrowej frakcji była utrudniona i wymagała potwierdzenia techniką Western blot. Obydwie techniki pozwalają również na uzyskanie danych ilościowych, poprzez analizę intensywności fluorescencji z wykorzystaniem programu *ImageJ* oraz ocenę intensywności prążków z zastosowaniem programu GelAnalyzer.

6.3.2.12.2. Analiza modyfikacji epigenetycznych

6.3.2.12.2.1. Analiza modyfikacji epigenetycznych DNA

Analizy metylacji DNA przeprowadzono poprzez immunofluorescencyjną detekcję 5-metylocytozyny (5-mC). Metoda ta pozwala na bezpośrednią ocenę poziomu i rozmieszczenia sygnału metylacji w jądrach komórek merystematycznych. Ponieważ metylacja DNA stanowi jeden z podstawowych mechanizmów epigenetycznej kontroli transkrypcji, jej analiza umożliwia określenie, czy czynniki stresowe lub fitotoksyczne zakłócają stabilność epigenomu i regulację ekspresji genów. Uzyskane dane pozwalają nie tylko na wykrycie zmian w globalnym poziomie metylacji, ale także na ocenę ich potencjalnego wpływu na aktywność transkrypcyjną i kondycję proliferujących komórek.

6.3.2.12.2. Analiza modyfikacji epigenetycznych histonów

Immunofluorescencyjna detekcja potranslacyjnie zmodyfikowanych histonów (H3K4Me2, H3K56Ac, H3T45Ph, H3K9Ac, H4K5Ac) z wykorzystaniem mikroskopii fluorescencyjnej umożliwia wizualizację rozmieszczenia wybranych znaczników epigenetycznych w jądrach komórek merystatycznych. Analizowane modyfikacje związane są zarówno z represją transkrypcji, procesami naprawy DNA, jak i aktywacją genów odpowiedzi stresowej. Ich ocena jakościowa i ilościowa przy użyciu programu ImageJ pozwala określić, czy czynniki fitotoksyczne zakłócają równowagę pomiędzy represją a aktywacją transkrypcji, a tym samym wpływają na regulację ekspresji specyficznych genów kontrolujących odpowiedź stresową.

6.3.2.12.3. Analiza procesu transkrypcji

Analiza procesu transkrypcji została przeprowadzona z wykorzystaniem inkorporacji 5-etynylourydyny (EU) do nowo syntetyzowanego RNA, wizualizowanej w mikroskopii fluorescencyjnej. Metoda pozwala na ocenę intensywności oraz rozmieszczenia sygnału, dostarczając informacji o globalnym poziomie transkrypcji w komórkach merystatycznych. Dzięki temu możliwe jest określenie wpływu czynników fitotoksycznych na podstawowy proces regulujący ekspresję genów odpowiedzialnych za proliferację i odpowiedź stresową.

6.3.2.12.4. Analiza ekspresji genów metodą RT-qPCR

Analizy ekspresji genów przeprowadzono metodą ilościowej reakcji łańcuchowej polimerazy z odwrotną transkrypcją (ang. *reverse transcription-quantitative polymerase chain reaction*, RT-qPCR). Metoda umożliwia dokładne określenie poziomu transkryptów wybranych genów w komórkach merystatycznych, pozwalając na ocenę zmian ekspresji w odpowiedzi na czynniki stresowe lub fitotoksyczne. qPCR dostarcza danych ilościowych, umożliwiając porównanie poziomu ekspresji między próbkami oraz identyfikację genów silnie regulowanych w warunkach eksperymentalnych.

6.3.2.12.5. Analiza struktury i aktywności jąder

Detekcja jąderkowych białek srebrochłonnych (AgNOR) została przeprowadzona przy użyciu mikroskopii świetlnej. Metoda ta umożliwia wizualizację aktywnych organizatorów jąderkowych oraz morfometryczną ocenę wielkości jąder, ściśle powiązanej zarówno z poziomem aktywności transkrypcyjnej, jak i biogenezą rybosomów. W przeciwieństwie do inkorporacji EU, która obrazuje jedynie globalną transkrypcję, analiza AgNOR umożliwia bardziej bezpośrednią ocenę aktywności biosyntezy rybosomów, procesu niezbędnego dla proliferacji komórek merystatycznych.

6.3.2.12.6. Analiza ultrastruktury komórek

Analizy zmian ultrastrukturalnych przeprowadzono przy użyciu transmisyjnej mikroskopii elektronowej (ang. *transmission electron microscopy*, TEM). Metoda umożliwia obserwację subkomórkowych organelli i struktur, takich jak mitochondria, plastydy, retikulum endoplazmatyczne czy jądro komórkowe, z rozdzielczością na poziomie nanometrów. Dzięki temu możliwe jest wykrycie drobnych zmian morfologicznych wywołanych działaniem czynników stresowych lub fitotoksycznych, które nie są widoczne w mikroskopii świetlnej, stanowiąc uzupełnienie danych biochemicznych i immunocytochemicznych.

6.4. Omówienie wyników

6.4.1. Praca 1 (Załącznik nr 1)

Szczęblewski, P.*; Wróblewski, M.; Borzyszkowska-Bukowska, J.; Bairamova, T.; Górską, J.; Laskowski, T.; Samulewicz, A.; Kosno, M.; Sobiech, Ł.; Polit, J. T.; Kukula-Koch, W. The Role of Centrifugal Partition Chromatography in the Removal of β -Asarone from *Acorus calamus* Essential Oil. *Sci Rep* **2022**, 12 (1), 22217. <https://doi.org/10.1038/s41598-022-26726-6>.

Prezentowana publikacja stanowi część szerszego projektu badawczego realizowanego przez zespół, którego jestem członkiem. Jedynie wybrany fragment tej pracy został włączony do rozprawy doktorskiej, ponieważ bezpośrednio odnosi się do jej celu, tj. charakterystyki chemicznej komercyjnie dostępnego olejku eterycznego z kłączy *Acorus calamus* (SEO) oraz określenia udziału poszczególnych izomerów azaronu. Wyniki te przedstawiono w publikacji w postaci Figury 2 i Tabeli 1 oraz Tabeli S1 pliku uzupełniającego (Supplementary File) jak również Tabeli 2 i Figury 3 (próbka S8).

Przeprowadzona analiza HPLC-ESI-QTOF-MS/MS komercyjnie dostępnego SEO, stosowanego w kolejnych badaniach, doprowadziła do identyfikacji 22 związków spośród około 40 pików obecnych na chromatogramie TIC (Total Ion Chromatogram). Zastosowany układ chromatograficzny jak i inne dobrane parametry aparatury okazały się wystarczające by otrzymać chromatogram z dobrze rozdzielonymi sygnałami niepolarnych składników SEO, a także wyraźne widma mas.

Zidentyfikowane związki chemiczne należą głównie do następujących grup:

1. Fenylopropanoidy (fenylopropeny) – β -, γ - i α -azaron, azaronaldehyd, metyleugenol, safrol
2. Aromatyczne ketony – izoakoramon, propioveratron, aspidinol
3. Seskwiterpeny – kalamenen, kalaren, α -cedren, shyobunon, akoronen, akorusol
4. Lignany/neolignany – surinamensyna
5. Inne – kwas glukonowy, tatarin A

Ponadto, półilościowa analiza azaronów zawartych w SEO z wykorzystaniem techniki HPLC-DAD pozwoliła na rozdzielenie i identyfikację trzech izomerów azaronu: α -, β - i γ -azaron. Biorąc pod uwagę względną zawartość procentową, stwierdzono, że β -azaron był głównym fenylopropanoidem (ponad 90% w badanym oleju komercyjnym), a następnie α -azaron i γ -azaron (Tabela 2, Figura 3, próbka S8).

Analiza ilościowa β -azaronu wykonana metodą HPLC-DAD z zastosowaniem wzorca zewnętrznego wykazała jego zawartość na poziomie 1,842 mg/ml (Tabela 2, Figura 3, próbka S8). Podobny wysoki udział tego związku opisywano również w olejkach z tataraku tetraploidalnego pochodzącego z Azji.

Pozostała część publikacji, dotycząca analizy komercyjnie dostępnych kłączy tataraku oraz próbek pobranych ze środowiska pod kątem zawartości β -azaronu, a także opracowania metody efektywnego usuwania β -azaronu z olejku, została zrealizowana w ramach dalszych prac zespołowych i nie wchodzi w zakres dysertacji. Stanowi ona jednak istotny element szerszej perspektywy badawczej, ponieważ po ocenie aktywności biologicznej pełnego składu SEO (z wysokim udziałem β -azaronu), która jest tematem niniejszej rozprawy doktorskiej zespół planuje w kolejnych etapach porównać te wyniki z efektami działania olejku pozbawionego β -azaronu. Opracowana w tej publikacji metoda usuwania β -azaronu otwiera drogę do takich porównań i poszerza kontekst analiz prezentowanych w niniejszej rozprawie.

6.4.2. Praca 2 (Załącznik nr 2)

Wróblewski, M.*; Piotrowska-Niczyporuk, A.; Ciereszko, I.; Gocek, N.; Żabka, A.; Szczepblewski, P.; Sobiech, Ł.; Saja-Garbarz, D.; Polit, J. T. Phytotoxicity and Bioherbicidal Potential of Sweet Flag (*Acorus calamus* L.) Essential Oil on Fabaceae and Brassicaceae Species. *Scientia Horticulturae* **2025**, *347*, 114180. <https://doi.org/10.1016/j.scienta.2025.114180>.

Praca poświęcona jest ocenie potencjału fitotoksycznego SEO wobec korzeni zarodkowych młodych siewek Fabaceae (*V. faba* var. *minor* i *L. luteus*) oraz Brassicaceae (*B. napus* i *A. thaliana*). W pierwszym etapie, bazując na wstępnie określonych zakresach stężeń zemulgowanego SEO (0,01–0,05% dla *V. faba*, *L. luteus* i *B. napus* oraz 0,004–0,009% dla *A. thaliana*), oceniono dynamikę wzrostu korzeni po 24-godzinnej inkubacji. Na podstawie uzyskanych wyników wyznaczono wartości stężenia IC₅₀ (0,03 % dla *V. faba*, 0,025% dla *L. luteus*, 0,01% dla *B. napus* oraz 0,005% dla *A. thaliana*), które pozwoliły ustalić porównywalny poziom stresu dla wszystkich gatunków, odpowiadający 50% zahamowaniu pierwotnego wzrostu korzeni. Tak przygotowany model eksperymentalny, stosowany we wszystkich kolejnych analizach umożliwił badanie mechanizmów działania SEO i ocenę reakcji fizjologicznych, cytologicznych oraz molekularnych w warunkach umiarkowanego stresu, wystarczającego do uruchomienia reakcji obronnych i adaptacyjnych, lecz nie prowadzącego do śmierci siewek. Jednocześnie stworzył podstawy do oceny, czy reakcje roślin mają charakter jednolity, specyficzny dla gatunku, czy też wykazują wzorce wspólne dla danej rodziny.

W kolejnym etapie przeanalizowano zmiany towarzyszące redukcji wzrostu korzeni. Wykazano, że oprócz zahamowania wzrostu korzeni o 50%, SEO powodował również wyraźny spadek świeżej i suchej masy (FW/DW), wskazując na obniżoną syntezę biomasy oraz redukcję nawodnienia tkanek. Równocześnie odnotowano spadek żywotności komórek, wykazany barwieniem błękitem Evansa, oraz uszkodzenia błon komórkowych, potwierdzone zwiększonym wyciekiem jonów w pomiarach konduktometrycznych. Chociaż zahamowanie wzrostu korzeni było porównywalne, spadek biomasy różnił się między gatunkami – największy wystąpił u *L. luteus* i *A. thaliana*, a najmniejszy u *V. faba* i *B. napus*. Mimo tych gatunkowo specyficznych różnic, ogólny wzorzec reakcji wskazał na większą odporność przedstawicieli Fabaceae w porównaniu z Brassicaceae, gdyż rośliny z tej rodziny zachowały wyższą żywotność komórek i wykazywały mniejsze uszkodzenia błon. Obserwacje te odzwierciedlają odpowiedź zarówno gatunkowo specyficzną, jak i zróżnicowaną na poziomie rodziny potwierdzając selektywną fitotoksyczność SEO.

Zaburzenia w integralności błon skłoniły do analizy stresu oksydacyjnego i produkcji reaktywnych form tlenu (ROS), wykrywanych w postaci H₂O₂. Równolegle analizowano odpowiedź i wydajność systemów antyoksydacyjnych, zarówno nieenzymatycznych (askorbinian, glutation, cysteina, γ-glutamylcysteina, α- i β-karoten), jak i enzymatycznych (dysmutazy ponadtlenkowej SOD, peroksydazy askorbinianowej APX, reduktazy glutationowej GR oraz katalazy CAT). Stwierdzono, że SEO istotnie zwiększa akumulację H₂O₂ u wszystkich gatunków. Choć największa akumulacja H₂O₂ wystąpiła w korzeniach Fabaceae, rośliny te uruchamiały efektywniejszy system antyoksydacyjny, co pozwalało skuteczniej ograniczać stres oksydacyjny wywołany przez SEO.

Ponieważ przyrost korzeni pierwotnych zależy od sprawności metabolizmu i dopływu hydrolizatów substancji odżywczych z liścieni – tkanki zapasowej kiełkujących nasion, wspierającej początkowy wzrost młodych siewek – zaburzenia oksydacyjne mogą istotnie oddziaływać na gospodarkę energetyczną komórek. Dlatego w ostatniej części pracy skoncentrowano się na ocenie wpływu SEO na metabolizm korzeni zarodkowych, wykorzystując kalorymetrię izotermiczną oraz analizy zawartości białek, cukrów i kwasów tłuszczowych.

Analiza emisji ciepła ujawniła odmienne strategie metaboliczne oraz wyraźne różnice między rodzinami roślin. Fabaceae reagowały kontrolowaną i zrównoważoną emisją energii, podczas gdy Brassicaceae wykazywały bardziej zróżnicowane odpowiedzi. U *B. napus* obserwowano stopniowy wzrost emisji ciepła względem kontroli, natomiast u *A. thaliana* wystąpiły wyraźne reakcje endoenergetyczne, wskazujące na zwiększony pobór energii. Wyniki te sugerują mniej efektywną adaptację metaboliczną Brassicaceae pod wpływem SEO w porównaniu z Fabaceae.

Analizy metaboliczne ujawniły spadek zawartości białek u Fabaceae i wzrost u Brassicaceae, a także gatunkowo specyficzne zmiany w poziomie monosacharydów oraz w profilu kwasów tłuszczowych: nasyconych (SFA), jednonienasyconych (MUFA) i wielonienasyconych (PUFA). Choć nie zaobserwowano spójnego wzorca odpowiedzi między rodzinami, najbardziej wyraźne zmiany dotyczyły Brassicaceae, różniąc się kierunkiem i skalą reakcji między gatunkami. Zestawienie tych wyników z pomiarami malonyldialdehydu (MDA), markera peroksydacji lipidów, wskazuje, że zmiany w składzie kwasów tłuszczowych u Fabaceae sprzyjają stabilności błon, podczas gdy u Brassicaceae mobilizacja lipidów i wyższa zawartość PUFA zwiększają podatność na peroksydację i uszkodzenia.

Ujawnione różnice w metabolizmie i w zakresie peroksydacji lipidów pod wpływem SEO mają też swoje źródło na poziomie nasion, które dostarczają do korzeni odmiennego „bagażu metabolicznego”. Fabaceae, bogate w skrobię i białka zapasowe, opierają wczesny metabolizm na węglowodanach i białkach, co sprzyja niższej produkcji ROS i lepszej integralności błon. Brassicaceae jako gatunki oleiste, mobilizują lipidy poprzez β -oksydację, która generuje więcej ROS, nasilając peroksydację lipidów i uszkodzenia błon. W konsekwencji, mimo wyższej akumulacji H_2O_2 , Fabaceae skuteczniej ograniczają peroksydację i wykazują większą odporność oksydacyjną, podczas gdy Brassicaceae charakteryzuje wyższa wrażliwość na SEO.

6.4.3. Praca 3 (Załącznik nr 3)

Wróblewski, M.*; Krajewski, K.; Gocek, N.; Żabka, A.; Polit, J. T. *Acorus calamus* L. Essential Oil Induces Oxidative Stress and DNA Replication Disruptions in Root Meristem Cells of Two Fabaceae and Two Brassicaceae Species. *IJMS* **2025**, 26 (10), 4715. <https://doi.org/10.3390/ijms26104715>.

W poprzednich badaniach wykazano, że SEO ogranicza wzrost korzeni zarodkowych i powoduje zaburzenia w strukturze błon oraz metabolizmie komórek. Ponieważ wzrost korzeni zależy w dużym stopniu od proliferacji w merystemie wierzchołkowej, w kolejnych etapach badań skoncentrowano się na analizie aktywności komórek merystematycznych. W niniejszej pracy oceniano bezpośrednio wpływ SEO w stężeniach IC_{50} specyficznych dla gatunków na przebieg interfazy, ze szczególnym uwzględnieniem replikacji DNA. Stres oksydacyjny wywołany przez SEO w komórkach merystematycznych zwizualizowano przy użyciu DAB oraz NBT. Zidentyfikowane reaktywne formy tlenu – nadtlenek wodoru i anionorodnik ponadtlenkowy – akumulowały się głównie w mitochondriach i plastydach, pozostawiając obszar jądra pozornie wolny od precypitatów barwnika. Pomimo tego w jądrze dochodziło do pęknięć nici DNA. W konsekwencji analiza cytofotometryczna wykazała gromadzenie się komórek w fazie G1 i spadek populacji komórek, których jądra inicjowały replikację, co potwierdziła ograniczona inkorporacja EdU. W komórkach replikujących proces przebiegał wolniej, czego dowodził słabszy niż w kontroli sygnał fluorescencyjny EdU w obrębie chromatyny. Charakterystyczny wzór znakowania jąder inkorporujących EdU umożliwia rozróżnienie kolejnych etapów fazy S: wczesnego – z równomiernym, luźno rozmieszczonym słabym sygnałem, zaawansowanego – z równomiernie rozmieszczonym gęstym, silnym sygnałem oraz późnego – z nierównomiernymi skupiskami fluorescencji (klastrami) odpowiadającymi replikacji heterochromatyny.

Zwiększony odsetek jąder wykazujących ostatni typ sygnału po zastosowaniu SEO wskazuje, że największe trudności w replikacji występowały w regionach heterochromatynowych. Wydłużenie fazy S i spowolnienie replikacji wiązało się z aktywacją mechanizmów naprawczych, co potwierdzono znakowaniem immunofluorescencyjnym histonu γ -H2AX. Liczne immunopoztywne ogniska w miejscach uszkodzeń DNA świadczą o aktywacji programów naprawczych. Pomimo obecności pęknięć, DNA nie ulegało znacznej degradacji - test TUNEL wykazał jedynie słaby sygnał w porównaniu z kontrolą pozytywną, a elektroforeza w żelu agarozowym nie ujawniła charakterystycznego obrazu „drabinki” typowego dla fragmentacji. U roślin w warunkach stresowych cykl komórkowy może ulegać przełączeniu w endoreplikację, stanowiącą strategię obronną umożliwiającą zwiększenie liczby kopii DNA i potencjału transkrypcyjnego. Badania cytofotometryczne nie wykazały jednak indukcji endocyklu pod wpływem SEO u żadnego z analizowanych gatunków. Wszystkie reagowały według podobnego schematu zmian w progresji fazy S, różniąc się jedynie nasileniem efektu. U Fabaceae odsetek komórek inicjujących replikację był niższy niż u Brassicaceae, co może odzwierciedlać silniejsze mechanizmy zabezpieczające przed ryzykiem błędów w podziale.

6.4.4. Praca 4 (Załącznik nr 4)

Wróblewski, M.; Gocek, N.; Żabka, A.; Polit, J. T.* Mitotic Disruption and Cytoskeletal Alterations Induced by *Acorus calamus* Essential Oil: Implications for Bioherbicidal Potential. *IJMS*. **2025**, 26 (18), 8933. <https://doi.org/10.3390/ijms26188933>.

Wykazane w poprzednich badaniach pęknięcia DNA, zaburzenia replikacji i zmiany proporcji komórek w interfazie musiały wpłynąć na aktywność mitotyczną, zależną od prawidłowego przebiegu wcześniejszych etapów cyklu komórkowego. Dlatego w niniejszej pracy przeanalizowano podziały komórkowe w obecności zemulgowanego SEO w stężeniach IC_{50} specyficznych dla badanych gatunków. Analiza indeksu mitotycznego ujawniła znaczną redukcję komórek dzielących się w merystemach wszystkich badanych gatunków o około 50-60%. Ocena indeksów fazowych wykazała natomiast największe nagromadzenie komórek w metafazie. W tym stadium obserwowano także najliczniejsze aberracje chromosomowe, obejmujące nierównomierną kondensację chromatyny, skupianie się telomerów, nieprawidłowe ustawienie chromosomów w płytce metafazowej, nadmierną kondensację, a także przedwczesną dekondensację chromosomów. W anafazie obserwowano nieprawidłowe rozchodzenie się chromatyd siostrzanych związane z występowaniem lepkich końców telomerowych, prowadzących do formowania mostków chromosomowych i mikrojąder. Ograniczenie indeksu mitotycznego oraz charakter stwierdzonych zaburzeń skłoniły do oceny zawartości głównego białka regulatorowego cyklu komórkowego, kinazy cyklinozależnej Cdc2 (CDKA), organizacji cytoszkieletu oraz epigenetycznych regulatorów mitozy: fosforylacji treoniny 3 i seryny 10 histonu H3, odpowiedzialnych zarówno za kondensację mitotyczną, jak i pośrednio za funkcjonowanie punktu kontrolnego wrzeciona oraz prawidłową separację chromatyd. Badania przeprowadzone na gatunkach będących przedstawicielami rodzin Fabaceae i Brassicaceae – *V. faba* i *B. napus* – wykazały spadek zawartości kinazy Cdc2, natomiast organizacja cytoszkieletu u wszystkich gatunków była silnie zaburzona. Mikrotubule były grubsze i intensywniej fluorescencyjne, co wskazuje na ich nadmierną stabilizację i silniejsze boczne wiązania. Pierścień preprofazowy utrzymywał się nietypowo długo, ograniczając dostępność tubuliny do tworzenia wrzeciona, natomiast w metafazie i anafazie włókna wrzeciona formowały gęsto upakowane, pęczkowe pasma. Podobne zaburzenia obserwowano w telofazie, gdzie fragmoplast wykazywał masywną strukturę. Filamenty aktynowe także ulegały agregacji, tworząc grube, silnie fluorescencyjne wiązki, choć ich ogólny układ przestrzenny pozostawał zachowany. Zaburzenia te wiązano z niedoborem Cdc2 i osłabieniem fosforylacji białek MAP odpowiedzialnej za

rozluźnienie bocznych wiązań mikrotubul, a także z upośledzeniem epigenetycznej regulacji mitozy. Stwierdzono bowiem nadmiernie utrzymującą się fosforylację H3T3 oraz niedobór fosforylacji H3S10. Zmiany te mogły wynikać zarówno z niedoboru kinazy Cdc2, jak i z blokowania przez SEO aktywności fosfataz PP1/2A, regulujących przebieg mitozy. Obserwowane zakłócenia mitozy różniły się nasileniem pomiędzy gatunkami, jednak we wszystkich przypadkach przebiegały według tego samego schematu, obejmującego podobne typy zmian w kolejnych etapach podziału komórkowego.

6.4.5. Praca 5 (Załącznik nr 5)

Wróblewski, M.; Gocek-Szczurtek, N.; Krajewski, K.; Żabka, A.; Polir, J. T.* Ultrastructural, signaling, and epigenetic responses to *Acorus calamus* L. essential oil in root meristems of Fabaceae and Brassicaceae seedlings – manuskrypt wysłany do czasopisma *The Plant Journal*.

Komórki merystematyczne, obok realizacji podstawowego zadania, jakim jest proliferacja, muszą jednocześnie sprawnie odbierać sygnały ze środowiska, aby odpowiednio reagować na zmieniające się warunki i dostosowywać do nich regulację cyklu komórkowego. Proces ten odbywa się m.in. poprzez epigenetyczne modyfikacje chromatyny oraz związane z nimi zmiany w ekspresji genów. Dlatego w niniejszej pracy, przy zastosowaniu zemulgowanego SEO w stężeniach IC₅₀ specyficznych dla badanych gatunków, skoncentrowano się na analizie sprawności szlaków sygnalizacji metabolicznej, w których główną rolę pełnią m.in. syntaza sacharozy (ang. *sucrose synthase*, SuSy), katalizująca rozkład sacharozy do fruktozy i UDP-glukozy (wykorzystywanej np. w biosyntezie celulozy i hemiceluloz ściany), heksokinaza (ang. *hexokinase*, HXK), fosforylująca glukozę pochodzącą np. z aktywności inwertazy, a także elementy kaskady kinaz MAP (MKK i MAPK p44/42) odpowiedzialne za przenoszenie sygnałów do jądra komórkowego. Równocześnie oceniano epigenetyczne modyfikacje chromatyny (DNA oraz histonów H3 i H4) charakterystyczne dla aktywacji bądź represji transkrypcji oraz dla procesów naprawy DNA, a także aktywność transkrypcyjną i ultrastrukturę komórek. Badania prowadzono głównie na przedstawicielach rodzin Fabaceae i Brassicaceae – *V. faba* i *B. napus*.

Wywołany przez SEO spadek aktywności HXK, która obok funkcji metabolicznej pełni też rolę sygnałną, został uwidocznił cytologicznie dzięki akumulacji barwnego produktu reakcji enzymatycznej (precypitatów NBT), lokalizujących się głównie w obszarach związanych z mitochondriami i plastydami, a także w postaci rozproszonej w cytoplazmie. Analiza ultrastrukturalna ujawniła, że obniżona aktywność HXK mogła mieć związek ze zmianami w organizacji tych organelli, w tym z przerwami w ciągłości ich błon, z którymi HXK pozostaje funkcjonalnie związana. W badaniach ekspresji genów zaobserwowano natomiast umiarkowany wzrost transkryptu HXK, jednak jego skala była mniejsza niż wzrost ekspresji SuSy. Wynik ten sugeruje, że w obliczu obniżonej aktywności HXK komórka nie zwiększa znacząco syntezy tego enzymu, lecz przesuwa metabolizm w stronę szlaków związanych z SuSy, które dostarczają UDP-glukozy. Taki kierunek adaptacji może odzwierciedlać potrzebę utrzymania integralności ściany komórkowej oraz równowagi metabolicznej w warunkach stresu oksydacyjnego.

W badaniach ekspresji genów stwierdzono jednocześnie wzrost transkryptu kinazy MKK, pełniącej w kaskadzie MAPK funkcję aktywatora kinazy MAP (p44/42). Równocześnie identyfikację ufosforylowanych, aktywnych izoform kinazy MAP (p44/42) przeprowadzono metodą immunofluorescencyjną w przypadku *V. faba* oraz techniką western blot u *B. napus*, dla którego obrazy mikroskopowe okazały się nieczytelne. W obu gatunkach stwierdzono wzrost poziomu aktywnej formy kinazy, a u *V. faba* dodatkowo wykazano jej jądrową lokalizację, wskazującą na przeniesienie sygnału do jądra komórkowego. Takie skorelowanie wzrostu transkryptu MKK z obecnością aktywnej MAPK podkreśla spójność reakcji całego szlaku sygnalizacyjnego w odpowiedzi na stres.

Konsekwencją aktywacji MAPK były identyfikowane immunofluorescencyjnie zmiany epigenetyczne w jądrze. Ogólnym efektem działania SEO był globalny wzrost metylacji DNA oraz modyfikacje histonów sprzyjające kondensacji chromatyny, a więc ograniczeniu jej dostępności i zmniejszeniu liczby miejsc aktywnych transkrypcyjnie. Obejmowały one: wzrost poziomu podwójnej metylacji H3K4, uznawanej u roślin za znacznik represji transkrypcji; spadek fosforylacji H3T45, normalnie ułatwiającej udostępnianie chromatyny dla procesów transkrypcji i replikacji (szczególnie widoczny u *B. napus*); oraz obniżenie globalnej acetylacji H4K5, zwykle związanej z rozluźnianiem struktury chromatyny.

Pomimo ogólnej przewagi modyfikacji epigenetycznych sprzyjających kondensacji chromatyny, zaobserwowano także lokalne zmiany ukierunkowane na aktywację transkrypcji w warunkach stresu. W szczególności wzbogacenie H4K5Ac w rejonach organizatorów jąderkowych sugerowało adaptacyjną próbę utrzymania transkrypcji rDNA i produkcji rybosomów. Równolegle wzrost poziomu H3K9Ac – zwykle zwiększającego się w regionach promotorowych genów aktywowanych w odpowiedzi na stres abiotyczny – wskazywał na możliwość selektywnej aktywacji genów obronnych mimo globalnej represji. Z kolei zmiany w profilu H3K56Ac, uznawanej za marker odpowiedzi na uszkodzenia DNA, przejawiające się zanikiem jednorodnego wyznakowania na rzecz wyraźnych ognisk sygnału, odzwierciedlały procesy naprawy DNA w miejscach uszkodzeń. W rezultacie odpowiedź epigenetyczna na SEO przyjmowała charakter dwutorowy: z jednej strony polegała na globalnym wyciszeniu chromatyny chroniącym integralność genomu, a z drugiej – na selektywnej aktywacji regionów niezbędnych do przetrwania w warunkach stresu oksydacyjnego.

W konsekwencji uruchomionych modyfikacji epigenetycznych stwierdzono ogólny spadek syntezy RNA, ujawniony metodą immunofluorescencyjną z zastosowaniem znacznika 5-etylnourydyny (EU). Wynik ten został potwierdzony analizą morfologiczną jąderka z wykorzystaniem techniki AgNOR – jąderka stały się mniejsze i bardziej jednorodnie wybarwione, co wskazywało na utratę zróżnicowania strukturalnego. Badania ultrastrukturalne potwierdziły te podejrzenia, ujawniając jąderka o zubożonym składniku włóknistym oraz obecność wakuoli jąderkowych w obrębie składnika ziarnistego, świadczących o obniżonej zawartości prerybosomów. Ponadto w otoczce jądrowej zaobserwowano rzadziej występujące pory jądrowe, co dodatkowo może wskazywać na zmniejszoną intensywność transportu między jądrem a cytoplazmą.

Obserwowane zmiany w remodelingu chromatyny i regulacji epigenetycznej pod wpływem SEO wskazują, że komórki merystatyczne aktywują konserwatywne mechanizmy adaptacyjne wspólne dla badanych przedstawicieli rodzin Fabaceae i Brassicaceae. Mimo podobnego schematu reakcji, intensywność i rozmieszczenie poszczególnych znaczników epigenetycznych wykazywały różnice międzygatunkowe, co może odzwierciedlać zróżnicowaną zdolność do radzenia sobie ze stresem oksydacyjnym. W szczególności merystemy *V. faba* wykazywały większą plastyczność chromatyny i sprawniejsze mechanizmy naprawcze, co prawdopodobnie umożliwiało skuteczniejsze utrzymanie funkcji komórkowych w warunkach działania SEO.

6.5. Wnioski ogólne

1. Olejek eteryczny z kłączy tataraku zwyczajnego (SEO) wykazuje wysoką selektywność działania, różnicując odpowiedź zarówno między gatunkami, jak i rodzinami roślin, co podkreśla jego potencjał w roli selektywnego bioherbicydu.
2. Uzyskane wyniki wskazują, że SEO może być szczególnie przydatny w kontroli chwastów z rodziny Brassicaceae w uprawach Fabaceae, wykorzystując różnice w sile reakcji pomiędzy tymi grupami roślin.
3. W przeciwieństwie do herbicydów syntetycznych, działających zazwyczaj poprzez pojedynczy cel molekularny, SEO oddziałuje wielokierunkowo. Równocześnie wpływa na integralność błon i stabilność DNA, na kluczowe procesy metaboliczne i mechanizmy redoks, na szlaki sygnalizacyjne oraz epigenetyczny remodeling chromatyny związany z transkrypcją i replikacją. Ponadto modyfikuje aktywność regulatorów cyklu komórkowego i organizację cytoszkieletu.
4. Systemowy charakter działania SEO zwiększa jego skuteczność i jednocześnie ogranicza ryzyko rozwoju odporności w populacjach chwastów, co czyni go szczególnie obiecującym narzędziem w rolnictwie zrównoważonym.

6.5.1. Wnioski – Praca 1 (Załącznik nr 1)

1. Skład olejku eterycznego *A. calamus* obejmuje głównie seskwiterpeny oraz fenylopropanoidy, spośród których dominują azarony, a najliczniejszy jest β -azaron.

6.5.2. Wnioski – Praca 2 (Załącznik nr 2)

1. SEO wykazuje zróżnicowaną fitotoksyczność wobec siewek Fabaceae i Brassicaceae, co potwierdza istotne różnice wartości IC_{50} . Efekt ten wskazuje na potencjał selektywnego wykorzystania olejku w rolnictwie, zwłaszcza w ochronie upraw Fabaceae przed chwastami z rodziny Brassicaceae.
2. Mechanizm działania SEO związany jest przede wszystkim z indukcją reaktywnych form tlenu (ROS) w korzeniach zarodkowych, prowadzącą do destabilizacji błon komórkowych. W odpowiedzi rośliny uruchamiają zarówno nieenzymatyczne (askorbinian, glutation, karotenoidy), jak i enzymatyczne (SOD, APX, GR, CAT) mechanizmy antyoksydacyjne, co potwierdza aktywację kompleksowej reakcji obronnej.
3. U roślin z rodziny Fabaceae odnotowano najwyższą akumulację ROS, co wskazuje na silny stres oksydacyjny. Jednak gatunki te efektywnie aktywowały mechanizmy antyoksydacyjne, dzięki czemu ograniczyły degradację błon i utrzymały wyższą żywotność komórek.
4. U gatunków Brassicaceae akumulacja ROS była niższa, lecz słabsze mechanizmy ochronne skutkowały poważniejszymi zaburzeniami metabolicznymi — wzrostem emisji ciepła (kalorymetria izotermiczna), nasileniem peroksydacji lipidów, większym uszkodzeniem błon komórkowych, większym wyciekami elektrolitów i wyraźnym spadkiem żywotności komórek.
5. SEO wywołał także gatunkowo specyficzne zmiany metaboliczne, odzwierciedlone zarówno w poziomie aktywności metabolicznej mierzonej kalorymetrią izotermiczną, jak i w różnicach w zawartości białek, cukrów i kwasów tłuszczowych. Zaobserwowane różnice mogą być częściowo związane z odmiennym składem rezerw nasiennych i strategiami kiełkowania poszczególnych gatunków, co sugeruje, że metabolizm węglowodanów i białek sprzyjał lepszemu zachowaniu integralności błon (*V. faba*, *L. luteus*), podczas gdy metabolizm lipidowy

u gatunków oleistych (*B. napus*, *A. thaliana*) mógł wiązać się z większą podatnością na stres oksydacyjny.

6. Uwzględniając wyższą tolerancję Fabaceae wobec działania SEO, zarówno na poziomie reakcji biochemicznych analizowanych przy IC_{50} , jak i przyrostu korzeni w szerokim spektrum stężeń, można wnioskować, że dawki skuteczne w ograniczaniu wzrostu Brassicaceae mogą oszczędzać Fabaceae, a w skrajnych przypadkach nawet sprzyjać ich rozwojowi.

6.5.3. Wnioski – Praca 3 (Załącznik nr 3)

1. SEO indukuje silny stres oksydacyjny w komórkach merystemów korzeni zarodkowych wszystkich badanych gatunków, przy czym ROS koncentrują się głównie w organellach cytoplazmatycznych (mitochondriach i plastydach).
2. SEO oraz generowany stres oksydacyjny wywołują pęknięcia nici DNA w komórkach merystematycznych wszystkich badanych gatunków, przy czym komórki rozpoznają uszkodzenia i aktywują mechanizmy naprawcze, o czym świadczy immunopozytywny sygnał γ -H2AX.
3. Zaburzenia struktury DNA zakłócają proporcje komórek w kolejnych etapach interfazy, prawdopodobnie w wyniku aktywacji punktu kontrolnego G1/S, prowadząc do zwiększenia populacji komórek w fazie G1 i zmniejszenia liczby komórek podejmujących replikację. Efekt ten jest bardziej nasilony u Fabaceae, u których mniej komórek inicjuje replikację w stosunku do kontroli, niż w przypadku Brassicaceae.
4. Dynamika procesu replikacji DNA jest ograniczona u wszystkich badanych gatunków, co objawia się słabszym włączaniem EdU i może wynikać z mniejszej liczby aktywnych replikonów lub wolniejszego tempa ich pracy.
5. Zwiększona subpopulacja komórek replikujących heterochromatynę w późnej fazie S, identyfikowana poprzez klastrowy charakter znakowania, wskazuje na utrudnienia w naprawie lub replikacji związane z wysokim stopniem kondensacji tych obszarów DNA.
6. Pomimo obserwowanej genotoksyczności, stres wywołany przez SEO w stężeniu IC_{50} nie prowadzi do rozległej fragmentacji DNA, co potwierdzają słabo pozytywny wynik testu TUNEL oraz elektroforeza DNA w żelu agarozowym.
7. Komórki nie przetaczają cyklu komórkowego na endoreplikację w odpowiedzi na uszkodzenia DNA wywołane przez SEO, jak potwierdzają pomiary całkowitej zawartości DNA.
8. Zakłócenia replikacji DNA wykazują specyfikę gatunkową pod względem nasilenia, ale nie rodzaju odpowiedzi – wszystkie badane gatunki prezentują podobny schemat zmian w progresji fazy S.

6.5.4. Wnioski – Praca 4 (Załącznik nr 4)

1. SEO redukuje odsetek komórek mitotycznych w merystemach korzeni zarodkowych wszystkich badanych gatunków. Efekt ten wynika zarówno z nagromadzenia komórek w fazie G1, spowodowanego zaburzeniami replikacji wywołanymi pęknięciami DNA, jak i z przedłużenia fazy S wskutek aktywacji mechanizmów naprawczych. Prawdopodobne jest również zaangażowanie punktu kontrolnego G2/M, który – przy obniżonej zawartości kinazy Cdc2, potwierdzonej metodą Western blot – ogranicza licencjonowanie komórek do podziału. Wyniki te wskazują, że SEO ingeruje w molekularne mechanizmy regulacji cyklu komórkowego.
2. SEO zmienia procentowy udział komórek w poszczególnych etapach mitozy u wszystkich badanych gatunków, powodując szczególnie wzrost subpopulacji w metafazie oraz

- powstawanie aberracji chromosomowych, co związane jest z nadmierną stabilizacją mikrotubul i filamentów aktynowych oraz ograniczoną dynamiczną reorganizacją wrzeciona kariokinetycznego i fragmoplastu.
3. Nadmierna stabilizacja cytoszkieletu indukowana przez SEO, udokumentowana w analizach immunofluorescencyjnych, wynika prawdopodobnie z zaburzeń regulacji aktywności białek MAP związanych z mikrotubulami. Przy obniżonej zawartości kinazy Cdc2 białka te nie podlegają sprawnej fosforylacji, ograniczającej wzajemne boczne wiązanie mikrotubul w metafazie. Skutkuje to nieprawidłowym podpinaniem mikrotubul do kinetochorów, ograniczoną dynamiczną niestabilnością mikrotubul oraz aktywacją punktu kontrolnego wrzeciona, prowadzącą do nagromadzenia komórek w metafazie.
 4. Zaburzenia separacji chromatyd wywołane przez SEO są konsekwencją nie tylko zakłóceń w funkcjonowaniu cytoszkieletu, lecz także zaburzeń epigenetycznej regulacji mitozy. Potwierdzono to immunofluorescencyjną detekcją fosforylacji treoniny 3 i seryny 10 histonu H3 (H3T3, H3S10), modyfikacji odpowiadających za funkcjonowanie punktu kontrolnego wrzeciona (SAC) i promujących pośrednio przejście metafaza/anafaza.
 5. Przedłużona i nasilona fosforylacja H3T3 pod wpływem SEO mogła utrudniać rekrutację kompleksu CPC/Aurora B do chromosomów, co zakłócało mechanizmy korygujące błędy w połączeniach kinetochor-mikrotubule i prowadziło do wtórnej aktywacji punktu kontrolnego wrzeciona.
 6. Osłabiona fosforylacja H3S10 obserwowana w obecności SEO, niezbędna dla kondensacji chromosomów mitotycznych, uwalniania kohezyny i prawidłowego rozdzielania chromatyd mogła być skutkiem zarówno zaburzonej rekrutacji kompleksu CPC/Aurora B, jak i ograniczonej zawartości kinazy Cdc2, aktywującej Aurora B.
 7. Nasilona, rozprzestrzeniona i przedłużona w czasie fosforylacja H3T3 w chromosomach mitotycznych silnie sugeruje, że SEO ogranicza również aktywność fosfataz PP1/2A, odpowiedzialnych za usuwanie grup fosforanowych z H3T3, a także z kinazy Haspin, która wprowadza tę modyfikację, wyłączając tym samym jej prawidłową regulację czasową.
 8. Zakłócenia przebiegu mitozy wykazują specyficzne różnice gatunkowe pod względem intensywności, ale nie rodzaju odpowiedzi – badane gatunki prezentują podobny schemat zmian w kolejnych etapach podziału komórkowego.

6.5.5. Wnioski – Praca 5 (Załącznik nr 5)

1. SEO zaburza sygnalizację metaboliczną zależną od heksokinazy (HXK), ograniczając jej aktywność enzymatyczną i w konsekwencji zmniejszając dostępność metabolitów cukrowych dla dalszych procesów metabolicznych. Zjawisko to może być związane z uszkodzeniami strukturalnymi błon, wykazanymi w mikroskopii elektronowej. Powstały sygnał odzwierciedla deficyt energetyczny, który może ograniczać zdolność komórek merystematycznych do proliferacji.
2. SEO prowadzi do wzmożonej aktywacji kaskady MAPK (p44/42), co potwierdzono zwiększoną akumulacją ufosforylowanych form tych kinaz w przedziale chromatynowym. Sugeruje to, że sygnały stresowe są przekazywane bezpośrednio do jądra, gdzie wpływają na funkcje chromatyny.
3. Sygnały przekazywane do jądra pod wpływem SEO inicjują remodelowanie chromatyny mające na celu utrzymanie homeostazy i zapewnienie przeżycia komórek. Proces ten manifestuje się globalną represją transkrypcji wynikającą z ograniczonej dostępności energii oraz

- specyficznymi modyfikacjami epigenetycznymi sprzyjającymi kondensacji chromatyny: wzrostem metylacji DNA, wzrostem poziomu H3K4Me2, spadkiem fosforylacji H3T45Ph oraz globalnym obniżeniem poziomu H4K5Ac.
4. Równolegle stres indukowany przez SEO i związane z nim uszkodzenia DNA aktywują adaptacyjne modyfikacje histonów, które pozwalają na selektywną odpowiedź transkrypcyjną i naprawę genomu. Należą do nich acetylacja H4K5 i H3K9, umożliwiającą aktywację genów odpowiedzi na stres, oraz acetylacja H3K56, sprzyjająca relaksacji chromatyny w obszarach wymagających efektywnej naprawy DNA.
 5. W odpowiedzi na zahamowanie HXK w warunkach stresu indukowanego przez SEO, komórki merystematyczne wydają się przeprogramowywać aktywność transkrypcyjną poprzez wzmocnienie alternatywnych szlaków metabolicznych. Obejmuje to podwyższoną transkrypcję syntazy sacharozy (SuSy), dostarczającą UDP-glukozę w odpowiedzi na zmniejszoną dostępności heksoz ze szlaków zależnych od HXK, przy jednoczesnym wzroście poziomu transkryptów HXK, co sugeruje próbę przywrócenia pierwotnej ścieżki detekcji cukrów i utrzymania przepływu węglowodanów w warunkach stresu.
 6. Zaobserwowane wzmocnienie sygnalizacji MAPK w odpowiedzi na stres wywołany SEO jest prawdopodobnie wspierane nie tylko przez zwiększoną fosforylację MAPK (p44/42), ale również przez podwyższoną transkrypcję kinaz nadrzędnych MAPK (MKK). Taka skoordynowana regulacja sugeruje, że komórki merystematyczne wzmacniają całą kaskadę MAPK, zapewniając solidną sieć sygnalizacyjną reagującą na stres, która przekazuje sygnały środowiskowe i metaboliczne do jądra.
 7. SEO nie zakłóca funkcjonowania maszyneryi remodelującej chromatynę interfazową, lecz aktywuje ją w sposób skoordynowany. Uzyskane zmiany epigenetyczne wskazują na kontrolowaną odpowiedź komórki, a nie na przypadkowe rozregulowanie procesów remodelingu.
 8. Mechanizm, za pomocą którego SEO moduluje strukturalne i funkcjonalne właściwości chromatyny, wydaje się być zachowany wśród badanych gatunków z rodzin Fabaceae i Brassicaceae, przy czym pewne wykazane różnice międzygatunkowe w poziomach i wzorcach dystrybucji określonych znaczników epigenetycznych, wskazują na większą plastyczność adaptacyjną Fabaceae w odpowiedzi na stres.

6.6. Bibliografia

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7. Dorobek naukowy

7.1. Spis publikacji niewchodzących w skład rozprawy doktorskiej

1. Żabka, A.; Winnicki, K.; Polit, J. T.; **Wróblewski, M.**; Maszewski, J. Cadmium (II)-Induced Oxidative Stress Results in Replication Stress and Epigenetic Modifications in Root Meristem Cell Nuclei of *Vicia faba*. *Cells* **2021**, *10* (3), 640. <https://doi.org/10.3390/cells10030640>.

7.2. Doniesienia konferencyjne

7.2.1. Wystąpienia ustne

1. 5th International Conference of Cell Biology jako współautor prezentacji *Effect of Candicidin D on plants differing in the structure of phytosterols* zorganizowana na Uniwersytecie Jagiellońskim w Krakowie w dniach 10 – 12 maja 2019 r. autorzy: **Mateusz Wróblewski**, Konrad Winnicki, Paweł Szczęblewski, Justyna Polit
2. II Ogólnopolska Konferencja Naukowa Rośliny w naukach medycznych i przyrodniczych zorganizowana w Lublinie w dniu 24 maja 2019 r. jako **autor prezentacji** *Wdrożenie i optymalizacja hodowli Arabidopsis thaliana in vitro jako modelu do oceny wpływu kandycydyny D na rośliny*.

7.2.2. Prezentacje plakatowe

1. 10th International Symposium on Root Development organizowanego przez Vlaams Instituut voor Biotechnologie (VIB) w Gandawie (Belgia) w dniach 15 – 18 maja 2023 r. jako współautor plakatu: *The potential of plant secondary metabolites as sustainable pesticides: assessing the impact of emulsified sweet flag essential oil on root development of faba bean and rapeseed* autorzy: **Mateusz Wróblewski**, Natalia Gocek, Konrad Winnicki, Aneta Żabka
2. 10th International Symposium on Root Development organizowanego przez Vlaams Instituut voor Biotechnologie (VIB) w Gandawie (Belgia) w dniach 15 – 18 maja 2023 r. jako współautor plakatu: *Evaluation of the protective potential of thyme oil towards embryonic root meristem cells of Vicia faba var. minor exposed to cadmium toxicity* autorzy: Natalia Gocek, **Mateusz Wróblewski**, Aneta Żabka, Justyna Polit
3. VIII Ogólnopolska Konferencja Doktorantów Nauk o Życiu Bioopen zorganizowana na Wydziale Biologii i Ochrony Środowiska Uniwersytetu Łódzkiego w dniach 13 – 14 kwietnia 2023 r. jako współautor plakatu *Ocena selektywnego działania olejku eterycznego z tataraku zwyczajnego na kiełkowanie bobowatych i kapustowatych* autorzy **Mateusz Wróblewski**, Natalia Gocek, Alicja Piotrowska-Niczyporuk, Alina Dubis, Aneta Żabka, Iwona Ciereszko, Justyna Polit
4. VIII Ogólnopolska Konferencja Doktorantów Nauk o Życiu Bioopen zorganizowana na Wydziale Biologii i Ochrony Środowiska Uniwersytetu Łódzkiego w dniach 13 – 14 kwietnia 2023 r. jako współautor plakatu *Modyfikacje epigenetyczne towarzyszące stresowi oksydacyjno-replikacyjnemu generowanemu przez metale ciężkie w komórkach roślinnych oraz potencjał protekcyjny olejku tymiankowego* autorzy: Natalia Gocek, **Mateusz Wróblewski**, Aneta Żabka, Justyna Polit
5. V Ogólnopolska Konferencja Doktorantów Nauk o Życiu Bioopen zorganizowana na Wydziale Biologii i Ochrony Środowiska Uniwersytetu Łódzkiego w dniach 30 – 31 maja 2019 r. jako współautor plakatu *Kandycydyna D zmienia poziom aktywności oraz lokalizację komórkową*

- kinaz MAP w korzeniach siewek Vicia faba subsp. Minor* autorzy: **Mateusz Wróblewski**, Konrad Winnicki, Paweł Szczęblewski, Justyna Polit
6. Ogólnopolska Konferencja Naukowa pt. Wyzwania doktorantów i doktorów wobec współczesnej nauki, zorganizowana w Warszawie w dniu 8 grudnia 2018 r. jako współautor plakatu: *Wpływ kandycydyny D jako potencjalnego fungicydu na rozwój siewek roślin różniących się budową chemiczną fitosteroli*, autorzy: **Mateusz Wróblewski**, Paweł Szczęblewski, Justyna Polit
 7. VII Konferencja Biologii Molekularnej zorganizowana przez SKN Młodych Biofizyków na Wydziale Biologii i Ochrony Środowiska Uniwersytetu Łódzkiego w dniach 12 – 14 kwietnia 2018 r. jako współautor plakatu *Kandycydyna D zmienia poziom fosforylacji kinaz MAP oraz zawartość białek PIN2 w korzeniach zarodkowych rzepaku*, autorzy: **Mateusz Wróblewski**, Konrad Winnicki, Aneta Żabka, Justyna Polit
 8. Konferencja Młodych Naukowców nt. Biologia, chemia i środowisko - spojrzenie młodych naukowców zorganizowanej w Krakowie w dniu 9 grudnia 2017 r. jako współautor plakatu *Wpływ kandycydyny D, potencjalnego środka ochrony roślin, na proliferację oraz rozwój siewek rzepaku*, autorzy: **Mateusz Wróblewski**, Konrad Winnicki, Aneta Żabka, Paweł Szczęblewski, Łukasz Sobiech, Justyna Polit
 9. III Ogólnopolska Konferencja Doktorantów Nauk o Życiu Bioopen zorganizowana na Wydziale Biologii i Ochrony Środowiska Uniwersytetu Łódzkiego w dniach 11 – 12 maja 2017 r. jako współautor plakatu *Wpływ kandycydyny D na wzrost siewek rzepaku* autorzy: **Mateusz Wróblewski**, Paweł Szczęblewski, Aneta Żabka, Konrad Winnicki, Justyna Polit
 10. VI Konferencja Biologii Molekularnej zorganizowana przez SKN Młodych Biofizyków na Wydziale Biologii i Ochrony Środowiska Uniwersytetu Łódzkiego w dniach 6 – 7 kwietnia 2017 r. jako współautor plakatu *Makrolidy polienowe jako potencjalne środki ochrony roślin*, autorzy: **Mateusz Wróblewski**, Justyna Polit

7.3. Działalność naukowa

7.3.1. Projekty badawcze

1. Doktoranckie Granty Badawcze, *Ocena fitotoksyczności i selektywności olejku tatarakowego oraz ustalenie docelowych miejsc jego oddziaływania w nasionach i siewkach dwóch gatunków uprawnych (Vicia faba var. minor L. i Lupinus luteus L.) oraz segetalnych (Brassica napus L. i Arabidopsis thaliana L.)*; **kierownik projektu**, instytucja finansująca: Uniwersytet Łódzki, 9/DGB/IDUB/2022, okres realizacji: styczeń 2022 r. – marzec 2025 r.
2. Studenckie Granty Badawcze III edycja, *Wdrożenie i optymalizacja hodowli Arabidopsis thaliana in vitro jako modelu do oceny wpływu kandycydyny D na rośliny*; **kierownik projektu**, instytucja finansująca: Uniwersytet Łódzki, okres realizacji: marzec – wrzesień 2019 r.
3. Studenckie Granty Badawcze II edycja, *Wpływ kandycydyny D jako potencjalnego fungicydu na rozwój siewek roślin różniących się budową chemiczną fitosteroli*; **kierownik projektu**, instytucja finansująca: Uniwersytet Łódzki, okres realizacji: lipiec – grudzień 2018 r.
4. Studenckie Granty Badawcze I edycja, *Wpływ kandycydyny D, potencjalnego środka ochrony roślin, na proliferację komórek oraz wzrost siewek rzepaku*; **kierownik projektu**, instytucja finansująca: Uniwersytet Łódzki, okres realizacji: lipiec 2017 r. – styczeń 2018 r.

7.3.2. Staże zagraniczne

1. Staż w Uniwersytecie Ludwika i Maksymiliana w Monachium (Niemcy) w ramach programu Erasmus+, tytuł projektu: *Unmasking the double face of sweet flag essential oil – evaluating the molecular impact at the transcriptional and chromatin levels in yellow lupine (Lupinus luteus L.)*, opiekun – prof. Claude Becker, okres realizacji: sierpień – październik 2023 r.
2. Staż w Max Planck Institute of Molecular Plant Physiology w Poczdamie (Niemcy) w ramach programu Erasmus+ tytuł projektu: *Unravelling the regulatory mechanisms of heat stress memory in Arabidopsis thaliana (at the transcriptional and protein quality control/autophagy levels)*, opiekunowie: Salma Balazadeh, PhD; Mastoureh Sedaghatmehr, Postdoc, okres realizacji: lipiec – wrzesień 2019 r.
3. Staż w DynaMo Center of Excellence na Uniwersytecie Kopenhaskim (Dania) w ramach programu Erasmus+, tytuł projektu: *Regulation of plant metabolism in response to nutrient deficiency*, opiekunowie: Meike Burow, Associate Professor; Verena Jeschke, PhD; Konrad Weber, PhD Fellow, okres realizacji: sierpień – wrzesień 2018 r.

7.3.3. Szkolenia

1. *Designing Project Management Projects for Sustainable Development*, zorganizowane przez Koç University Office of Learning and Teaching (KOLT), Koç University (Turcja) w dniu 14 marca 2025 r. w ramach sieci UNIC
2. *Western blot, technika, która da się zoptymalizować* zorganizowane przez firmę Agrisera w dniu 6 kwietnia 2022 r.
3. *New methods of confocal and two-photon confocal microscopy* prowadzone przez prof. Naoto Kawakami zorganizowane na Wydziale Biologii i Ochrony Środowiska Uniwersytetu Łódzkiego w dniach 4-12 czerwca 2019 r.
4. Zasady Dobrej Praktyki Laboratoryjnej oraz wymagania w Laboratorium Akredytowanym wg normy PN-EN ISO/IEC 17025 zorganizowane w dniach 19-20 czerwca 2018 r.
5. *HPLC-kurs podstawowy* zorganizowany w dniach 15-17 czerwca 2018 r.
6. Szkolenie w zakresie przygotowywania i rozliczania wniosków o środki finansowe na badania naukowe w ramach programu Studenckie Granty Badawcze zorganizowane na Uniwersytecie Łódzkim w dniu 25 maja 2017 r.
7. Kurs *Writing in the Sciences* zorganizowany przez Stanford University Online w dniu 22 kwietnia 2017 r.

7.4. Działalność organizacyjna

7.4.1. Działalność na rzecz Uniwersytetu Łódzkiego

1. Przedstawiciel Doktorantów w Radzie Wydziału Biologii i Ochrony Środowiska Uniwersytetu Łódzkiego w roku akademickim 2024/2025
2. Członek Komisji Dyscyplinarnej dla Doktorantów na kadencję 2021 – 2024 r.

7.4.2. Działalność na rzecz promocji nauki, Wydziału oraz Uniwersytetu

1. Udział w organizacji warsztatów w ramach akcji Uniwersytet Zawsze Otwarty w okresie 2023-2025 r.
 - *Centrum dowodzenia komórki - jądro komórkowe i mitochondria*

- *Skazani na siebie – organizmy, które stały się organellami*
 - *Księga szyfrów – materiał genetyczny*
 - *Tkanki roślinne – różnorodność i porozumienie podstawa sprawnego funkcjonowania organizmu*
 - *Atrybuty komórek roślinnych – ściana, wakuole i plastydy*
 - *Zaprojektuj swoją przygodę z mikroskopią fluorescencyjną*
2. Udział w organizacji warsztatów w ramach Instytutu Kreatywnej Biologii w okresie 2023-2025 r.
 - *Różnorodność komórek roślinnych w obiektywie*
 - *Tajemnice mikroświata komórek roślinnych*
 3. Udział w organizacji wydziałowego charytatywnego kiermaszu roślin *Weź roślinkę, nakarm psinkę... i kotka też* w okresie 2022 – 2024 r.
 4. Udział w organizacji Fascynującego Dnia Roślin (*Fascination of Plants Day*) w 2022 r.
 - *Wycieczka w makro- i mikroświat roślin – podróż z przewodnikiem*
 5. Udział w organizacji Nocy Biologów w okresie 2018 – 2024 r.
 - *Barwy Biosfery: komórkowy krajobraz roślin*
 - *Woda – życiodajny rozpuszczalnik dla barw przyrody - popłynij z nim poprzez komórki i tkanki roślin*
 - *Jak komórki roślin mogą zareagować na globalne zmiany środowiska*
 - *Naturalna mikroapteka podstawą piramidy zdrowego trybu życia*
 - *Barwny świat wnętrza komórek*
 6. Udział w organizacji Dnia Otwartego Wydziału Chemii i Wydziału Biologii i Ochrony Środowiska dnia 5 kwietnia 2024 r.
 7. Udział w spocie nakręconym we współpracy z KOLEO promującym wybór kolei jako ekologicznego środka transportu – data publikacji 13 maja 2024 r.
 8. Udział w organizacji XVII Festiwalu Nauki, Techniki i Sztuki w dniach 5 – 8 kwietnia 2017 r.
 - *Kolorowy świat roślin w mikroskopie*
 - *Rewolucje naukowe #Biofizyka*
 9. Udział w organizacji VI Konferencji Biologii Molekularnej zorganizowanej przez SKN Młodych Biofizyków na Wydziale Biologii i Ochrony Środowiska Uniwersytetu Łódzkiego w okresie 6 – 8 kwietnia 2017 r.

7.5. Nagrody i wyróżnienia

1. Nagroda Rektora za działalność na rzecz i dla dobra Uniwersytetu Łódzkiego za semestr letni roku akademickiego 2023/2024
2. Nagroda Rektora za działalność na rzecz i dla dobra Uniwersytetu Łódzkiego za semestr zimowy roku akademickiego 2023/2024
3. Wyróżnienie prezentacji plakatu *Ocena selektywnego działania olejku eterycznego z tataraku zwyczajnego na kiełkowanie bobowatych i kapustowatych* podczas VIII Ogólnopolskiej Konferencji Doktorantów Nauk o Życiu BioOpen w dniach 13 – 14 kwietnia 2023 r.
4. Nagroda Rektora za działalność na rzecz i dla dobra Uniwersytetu Łódzkiego w 2023 r.
5. Stypendium Rektora dla najlepszych studentów w roku akademickim 2018/2019

8. Załączniki

1. Praca 1 (Załącznik nr 1)
2. Praca 2 (Załącznik nr 2)
3. Praca 3 (Załącznik nr 3)
4. Praca 4 (Załącznik nr 4)
5. Praca 5 (Załącznik nr 5)
6. Oświadczenia współautorów (Załącznik nr 6 A-R)



OPEN

The role of centrifugal partition chromatography in the removal of β -asarone from *Acorus calamus* essential oil

Paweł Szczepblewski^{1✉}, Mateusz Wróblewski², Julia Borzyszkowska-Bukowska¹, Tetiana Bairamova¹, Justyna Górka¹, Tomasz Laskowski¹, Anna Samulewicz³, Michał Kosno¹, Łukasz Sobiech⁴, Justyna Teresa Polit² & Wirginia Kukula-Koch⁵

B-asarone is a phenylpropane derivative present in the rhizomes of *Acorus calamus*, that was proved to exhibit toxic effects in humans. Because of its presence the whole plant that is commonly used in traditional medicine for its sedative, anti-inflammatory, neuroprotective and other properties has limited application nowadays. In the study, qualitative and quantitative analysis of a collection of nine essential oil (EO) samples of European and Asian origin was performed. The final content of β -asarone in the tested samples ranged between 0.265 and 1.885 mg/mL. Having in mind a possible application of the EO as a biopesticide, this research aimed at the development of CPC-based purification protocol that could help remove β -asarone from EO. It was proved that the biphasic solvent system composed of n-hexane/EtOAc/MeOH/water, 9:1:9:1 (v/v/v/v) was capable of the removal of the toxic constituent in the CPC chromatograph operated in the ascending elution mode with 2200 rpm and a flow rate of 5 mL/min. The chromatographic analysis that lasted only 144 min effectively separated β -asarone (purity of 95.5%) and α -asarone (purity of 93.7%) directly from the crude *Acorus calamus* rhizome EO.

Maintaining high quality crop products parallelly with reduced synthetic pesticides content is the biggest challenge for the modern agriculture. The toxic pesticide residues accumulate in crop yields, soil and ground waters what triggers the development of pests' and weeds' resistance, but simultaneously, affects soil microbiome and well-being of people and animals. However, the plant derived products of the secondary metabolism which include, *inter alia*, volatile components/ essential oils (EO) usually recovered from plant matrix by hydrodistillation seem to be a promising alternative for the synthetic pesticides used so far. Additionally, the EOs were proved to be efficient both in the prevention and treatment of different diseases¹. Formerly their antibacterial, antifungal and insecticidal properties were discussed in the scientific literature². Furthermore, their strong allelopathic properties such as the influence on germination and seedling growth should not be underestimated. The richness and variety of EOs components affects their diversified mechanisms of action and consequently hampers the resistance development in pathogens, pests, and weeds towards the oils. In the end, the effortless biodegradability of EO does not pose a risk to the environment.

The EO that exhibits dualistic, and thus intriguing properties is the calamus oil. It is extracted from rhizomes of sweet flag (*Acorus calamus* L.) belonging to Araceae botanical family. The leading constituents of calamus EO are phenylpropane derivatives (biosynthesized via the shikimate pathway), including propenyl isomers: a mixture of *trans* form of α -asarone, *cis* form of β -asarone ((E)-/(Z)-1,2,4-trimethoxy-5-prop-1-enylbenzene) and an allylic derivative γ -asarone (1,2,4-trimethoxy-5-prop-2-enylbenzene) (see Fig. 1). Methyl Eugenol, (Z)-methylisoeugenol, and elemicin are the remaining phenylpropenes detected in the calamus oil. The second group of EO constituents are terpenes: monoterpenes (α - and β -pinene, camphene, myrcene, limonene) and

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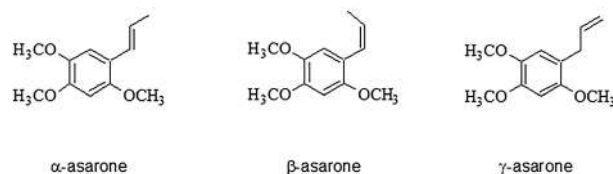


Figure 1. The isomers of asarone found in *Acorus calamus* EO.

sesquiterpenes (cadinene, acorone, acorenone, calamine). Also, the presence of tannins, choline, and fatty acids in its rhizomes was previously described^{3–6}.

The amount of phenylpropenes varies depending on the ploidy of *A. calamus* variant. In North America and in parts of Asia (Siberia) a diploid form ($2n = 2x = 24$) of the sweet flag may be found, which was proved to contain only 2% of EOs with no or trace amounts of β -asarone in its composition. *A. calamus* plants growing in Europe and temperate regions of Asia are primarily triploids ($2n = 3x = 36$) containing more EOs (3%) and little amounts of β -asarone (5–19%) in the EO. The third, tetraploid variety ($2n = 4x = 48$) that is most commonly used for the industrial production of calamus oil is widespread in East Asia, Japan and India and contains up to 6% of EOs and the largest amount of β -asarone (70–96%) in the EO from the rhizomes^{7,8}.

For many years sweet flag extracts remained substances of interest due to their medicinal properties. Calamus preparations exhibit neuroprotective, antidepressant, anticonvulsive, sedative, hypnotic, anxiolytic, memory enhancing, antiasthmatic, antihyperlipidemic, anticholestatic, antithrombotic, antiinflammatory, antioxidant, anticarcinogenic, antimicrobial and insecticidal properties^{9–12}. Even if the traditional usage of the plant in medicine, fragrance industry and foods has been wide, the exact *modus operandi* of calamus phytochemicals is still unexplored. Also, the contradictory reports on the toxicological safety of calamus oil for humans still need a final conclusion. Some authors mention cardiotoxicity, hepatotoxicity, reproductive toxicity, mutagenicity and carcinogenicity of β -asarone⁷. Based on the reported adverse effects, legal regulations for the use of β -asarone have been established. Within the European Union, the use of β -asarone as a pure substance or in the form of the tetraploid calamus essential oil for flavoring purposes is currently prohibited. In the United States of America, the use of calamus oils and its preparations is utterly disallowed. Several scientific reports on the therapeutic application of β -asarone, like memory enhancing properties¹³ or antimicrobial action¹⁴ are questionable in the light of the regulations described above. The observed tendency in the number of scientific manuscripts in the Scopus database shows a decrease in the number of articles concerning *Acorus calamus*, while the number of scientific papers on β -asarone itself stays on the same level.

Despite the toxicological data presented above, calamus EO still bears potential to be used as a biopesticide¹⁵. It was proved that calamus oil contains a number of important active substances (other than β -asarone) with a synergistic effect when in mixture^{16–19}. In the light of these findings, a good solution, safe for farmers and customers, might be to develop a method of effective removal of β -asarone from calamus oil aspiring to the role of a biopesticide—to be able to use it with no risk for poisoning of humans.

Classical isolation methods used to separate EOs components, like HPLC or TLC chromatography, even if they are offering several advantages like the reproducibility of results, automation, quantitative determination, short analysis time, they are not suitable for the analysis of all secondary metabolites or in the purification on semi- or preparative scale²⁰. One should mention here an irreversible adsorption on the chromatographic bed, which results in the sample loss and the damage of expensive preparative columns, tailing of compounds of interest on the stationary phase which decreases the purity of the isolates, or small loading capacities which limit the application of these techniques to the industrial scale isolation protocols. Also, the high consumption of solvents makes the classical separation process unprofitable and failing the requirements of green chemistry practice.

In the scientific literature there are only a few examples of β -asarone isolation. Previously, McGaw and co-investigators introduced a two-step fractionation (column chromatography and preparative thin layer chromatography) of the crude ethanol extract from *Acorus calamus* roots, rhizomes and leaves. In the proposed protocol they obtained 15.6 mg of the component from 1.14 g of extract¹⁴. In another work the same compound was purified from the rhizome volatile oil with chloroform: petroleum ether (4:1 v/v) using a silica gel chromatographic column¹³. The two mentioned above techniques were based on the application of classical chromatographic tools in the recovery of β -asarone and are bearing several limitations. The recovery rate and the loading capacity of these protocols was low, whereas the procedure tedious and the selectivity poor. Both techniques needed high volumes of solvents to be run, were time consuming, and the cited sources did not provide any information about the purity of the isolate. Based on these observations the authors of this manuscript found it important to search for a new isolation protocol that would provide high purity β -asarone directly from the crude EO sample to be either removed or isolated from EO. Easy scale-up, low solvents consumption and short analysis time would be preferred for future application in biopesticide production as well as in other calamus preparations.

Centrifugal Partition Chromatography (CPC) is a technique that is devoid of these drawbacks. This modern separation tool is the hydrostatic type of countercurrent chromatography, which uses the phenomenon of liquid–liquid partitioning of compounds between two non-miscible phases that stay at equilibrium²¹. One phase (stationary one) is retained in the column, while the other one (mobile phase) is pumped through it. Both immiscible liquids are in constant contact, emulsifying and separating repeatedly, creating the phenomenon of theoretical plates. The retention mechanism of stationary phase is determined by hydrostatic force, formed by the centrifugal field in the rotor in one-axis centrifuge^{22,23}. The separation of constituents of a mixture is obtained as

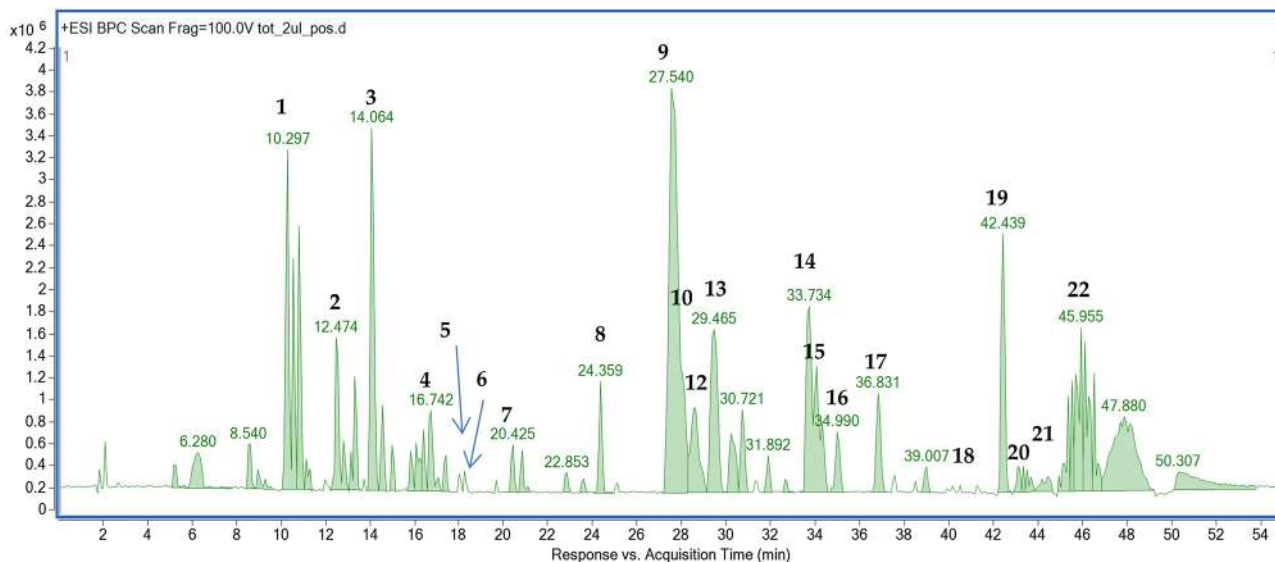


Figure 2. The total ion chromatogram (HPLC–MS) in positive ionization mode of the commercial essential oil sample obtained from the rhizomes of *Acorus calamus*.

a function of partition coefficient K_d , defined as the concentration of target compound in the stationary phase divided by the concentration in the mobile phase²⁴.

CPC is able to provide repetitive and selective separation conditions for the purification of single metabolites from plant extracts—also on preparative scale. High selectivity of applied methods, low solvent consumption, high sample loading capacity and lack of solid support that prevents sample loss is conducive to recover sufficient quantities of natural products even from complex plant extracts^{25,26}. However, the scientific literature on the analysis of essential oils by counter current chromatography is limited. Interestingly, according to our knowledge only four scientific works are focusing on the separation of essential oils using Centrifugal Partition Chromatography were traceable^{27–30}, while the remaining majority concerned the HSCCC technique. At this point it is worth mentioning that the CPC technique creates more advantageous conditions for a large-scale separations than the HSCCC technique.

Therefore the aim of this study is to develop precise single-step methodological conditions that would provide calamus oil free from β -asarone to be used on industrial scale. For this purpose CPC chromatography will be used to give hope for the successful exclusion of this potentially toxic phenylpropane derivative and possible application of the β -asarone-free EO as biopesticide in the future. Also, the determination of β -asarone content in the personally collected and commercially available EO samples will be performed with help of the HPLC-DAD and HPLC-ESI-QTOF-MS/MS techniques to confirm the actual content of the terpene of interest in the European and Asian samples and to emphasize the need for its removal.

Results and discussion

Essential oil composition by HPLC-ESI-QTOF-MS/MS. The performed HPLC-ESI-QTOF-MS/MS analysis of the EO samples led to the identification of 22 compounds out of ca. 40 signals present in the mass chromatogram. The applied instrument was sensitive enough to show clear mass spectra and sharp peaks of unpolar constituents of *Acorus calamus* rhizomes using a standard RP-18 chromatographic column. The composition of a sample EO and the list of tentatively identified components that was prepared in consideration with literature results on different *Acorus* spp. are presented in the Fig. 2, in Table 1 and also Table S1 (Supplementary File).

Among the representatives of different classes of compounds, asarones belonged to the most abundant components of the tested essential oils, with β -asarone as the leading compound. The identified constituents—terpenes and other asarone-related phenylpropanoids, were previously mentioned by other authors in *Acorus calamus* and *Acorus tatarinowii* species, as shown in Table 1.

Quantitative analysis of asarone content in EO by HPLC-DAD. The application of a liquid chromatography in the analysis of phenylpropanoids' content in the EO of *A. calamus* brought successful results. The HPLC-DAD based quantitative analysis of the asarones visualized the three forms of asarones: α -, β - and γ -asarone. These compounds were eluted from the column at 14.0, 11.5 and 12.8 min, respectively (see Fig. 7A). The quantitative analysis of asarones in the tested samples revealed marked differences between the samples. Considering the relative percentage content, β -asarone was found to be the major phenylpropanoid visible in the HPLC-DAD chromatogram, followed by α -asarone and γ -asarone in all tested samples (see Table 2). Interestingly, the total concentration of the β -asarone differed between the samples (see Fig. 3).

Its content was calculated as 1.32–1.41 mg/mL of the EO in the samples S1 and S2, 0.27 mg/mL in the sample S3, 0.19–0.41 mg/mL in the EOs from the commercially available rhizomes (samples S4–S6) and 1.75–1.89 mg/

No	Ion (+/−)	Rt (min)	Molecular formula	m/z calculated	m/z experimental	Delta (mmu)	DBE	MS/MS fragments	Proposed compound	References
1	[M+H] ⁺	10.3	C ₁₂ H ₁₆ O ₄	225.1121	225.1136	5.42	5	210, 193, 165	Isoacoramone isomer1	Sinha et al. ³¹
2	[M+H] ⁺	12.4	C ₁₂ H ₁₆ O ₄	225.1121	225.1131	− 4.3	5	210, 193, 165	Isoacoramone isomer2	Sinha et al. ³¹
3	[M+H] ⁺	14.1	C ₁₀ H ₁₂ O ₄	197.0808	197.0811	− 1.35	5	169,154,138	Asaronaldehyde	Jaiswal et al. ³²
4	[M+H] ⁺	16.7	C ₆ H ₁₂ O ₇	197.0656	197.0662	− 3.17	1	169, 154, 138	Gluconic acid	Strzepek-Gomolka et al. ³³
5	[M+H] ⁺	18.0	C ₁₁ H ₁₄ O ₃	195.1016	195.1014	0.88	5	–	Propioveratrone	Jaiswal et al. ³²
6	[M+H] ⁺	18.2	C ₁₇ H ₁₂ NO ₃	279.0890	279.0812	− 2.73	12.5	–	Tatarine A	Zhang et al. ³⁴
7	[M+H] ⁺	20.1	C ₁₀ H ₁₀ O ₂	163.0754	163.0760	− 3.97	6	–	Safrol	Jaiswal et al. ³²
8	[M+H] ⁺	24.6	C ₁₅ H ₂₂ O ₂	235.1693	235.1702	− 4.03	5	217, 179, 161	Acoronene	Yang et al. ³⁵
9	[M+H] ⁺	27.5	C ₇ H ₆ O ₃	209.1172	209.1163	4.42	5	168,153	2-Allyl-5-ethoxy-4-methoxyphenol isomer 1 (β-asarone)	Jaiswal et al. ³² Yang et al. ³⁶
10	[M+H] ⁺	28.3	C ₁₅ H ₂₂	203.1794	203.1799	− 2.34	5	161, 147, 133	Calamenene	Jaiswal et al. ³²
11	[M+H] ⁺	28.6	C ₁₁ H ₁₄ O ₂	179.1067	179.1071	− 2.49	5	164, 151	Methyl-eugenol	Jaiswal et al. ³²
12	[M+H] ⁺	28.6	C ₇ H ₆ O ₃	209.1172	209.1179	− 3.26	5	168, 153	2-Allyl-5-ethoxy-4-methoxyphenol isomer 2 (γ-asarone)	Jaiswal et al. ³² Yang et al. ³⁶
13	[M+H] ⁺	29.4	C ₇ H ₆ O ₃	209.1172	209.1183	− 5.19	5	194, 181	2-Allyl-5-ethoxy-4-methoxyphenol isomer 3 (α-asarone)	Jaiswal et al. ³² Yang et al. ³⁶
14	[M+H] ⁺	33.6	C ₁₂ H ₁₆ O ₄	225.1121	225.1136	− 6.54	5	210, 193, 165	Aspidinol	Jaiswal et al. ³²
15	[M+H] ⁺	33.9	C ₁₅ H ₂₂ O	219.1743	219.1753	− 4.39	5	177, 159, 121	Squamulosone isomer 1	Jaiswal et al. ³²
16	[M+H] ⁺	34.7	C ₂₂ H ₂₈ O ₆	389.1959	389.1972	− 3.44	9	–	Surinamensin	Jaiswal et al. ³²
17	[M+H] ⁺	36.8	C ₁₅ H ₂₂	203.1794	203.1811	− 8.27	5	161, 147, 133	Calamenene isomer	Jaiswal et al. ³²
19	[M+H] ⁺	41.4	C ₁₅ H ₂₄ O ₂	237.1849	234.1848	0.45	4	219, 135	Acorusnol	Yannai ³⁷
18	[M+H] ⁺	42.8	C ₁₅ H ₂₂ O	219.1743	219.1752	− 3.93	5	177, 137, 121	Squamulosone isomer 2	Jaiswal et al. ³²
20	[M+H] ⁺	42.4	C ₂₂ H ₂₄	205.1951	205.1953	− 1.09	4	165, 149, 125	Calarene	Loying et al. ³⁸
21	[M+H] ⁺	44.1	C ₂₂ H ₂₄	205.1951	205.1961	− 5.01	4	149, 121, 81	α-Cedrene	Jaiswal et al. ³²
22	[M+H] ⁺	45.9	C ₁₅ H ₂₄ O	221.1900	221.1920	− 9.12	4	153, 83	(+)-Shyobunone	Jaiswal et al. ³²

Table 1. The putative identification of secondary metabolites present in the essential oil obtained from the tested rhizome of *Acorus calamus*. DBE, double bond equivalent; delta, error of measurement; Rt, retention time; Ion, ionization mode.

Source	Rhizomes of natural accessions			Commercially available rhizomes			Commercially available EOs		
Code	S1	S2	S3	S4	S5	S6	S7	S8	S9
Asarone content [%]									
α	9.9	8.4	8.7	10.2	9.0	14.2	4.7	6.2	4.8
β	89.2	90.9	90.8	88.7	90.0	84.7	94.1	92.6	94.0
γ	0.8	0.8	0.5	1.1	1.0	1.2	1.2	1.2	1.1

Table 2. The relative amount [%] of asarones in the analysed EOs.

mL in the commercially available EOs from *A. calamus* (Samples S7–S9). Interestingly, the content of β-asarone differed depending on the place of collection of the rhizomes. The quantity of this compound in the Sample S3 that was collected in the central Poland in Malczew was significantly lower from the samples S1 and S2 (see Fig. 3). It could be explained by the presence of different ploidy plants on the area of Poland.

The obtained quantitative results for β-asarone in calamus oil are in line with some previously published data. A thorough study on its content in Finnish and Czech samples authored by Dušek et al.³⁹ proved that this phenylpropanoid was present in the oil at the quantity of 9.7–21.3% that resembles the concentration of 0.97–2.13 mg/mL. In general, the samples from Finland contained lower quantities of β-asarone in comparison to the Czech samples, but still its content was higher than in the herein investigated samples from the central Poland.

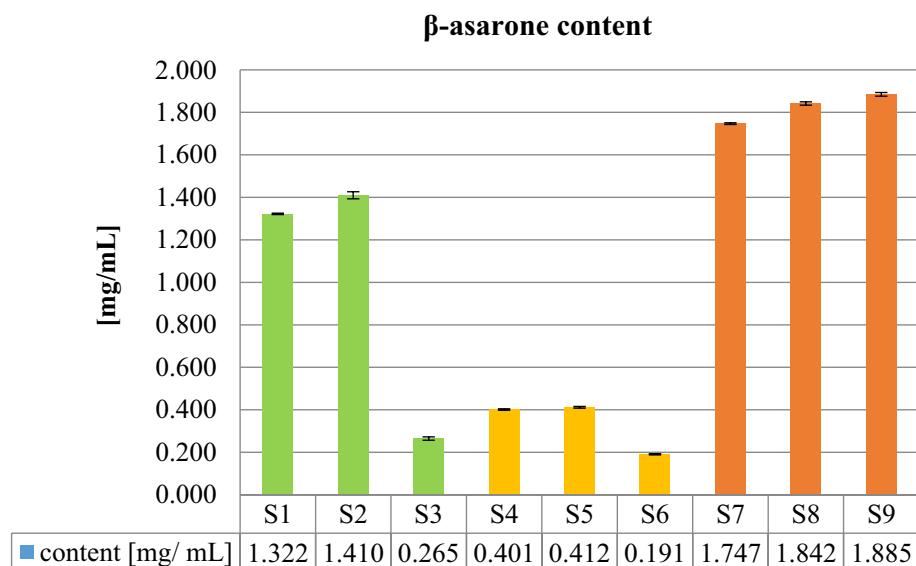


Figure 3. The β -asarone concentration in the analysed EOs.

Also, in the herein presented study the content of β -asarone was high in the commercially available EOs. The concentration of this phenylpropanoid in the samples S7–S9 resembled its content in the samples S1–S2.

The quantitative analysis of β -asarone content in the analysed samples brought to light an interesting issue, namely the geographical occurrence of different ploidy *A. calamus* plants. Even if Poland was expected to host triploids containing more EOs with small share of β -asarone, as mentioned in the introduction section, the quantitative analysis showed marked quantity of this component in the calamus oil similarly to the, widely distributed in Asia, tetraploid variety. The calculated β -asarone content is close to S9 sample of the Indian origin. Based on the obtained results, we could assume there are different ploidy plants in European grounds.

According to Raal and co-investigators⁴⁰ β -asarone was also the major constituent of the *A. calamus* rhizome essential oil that was obtained from the specimen grown in Estonia. The compound of interest constituted 85.3% in one sample—that was identified as a tetraploid sweet flag and 9.3–10.2% in other samples that were possibly triploids. These data confirmed the possibility to find in Europe different ploidy specimen growing in nature. The authors of this publication underlined that their data from 2016 mentioned for the first time the presence of β -asarone-rich chemotype of sweet flag in Europe. For sure, these specimens can also grow on the territory of Poland, as proved in our study.

Chemometric assessment. Since various extracts from *Acorus calamus* were collected at different dates and using different sources and techniques, for chemometric analysis it was decided to examine the relative concentrations of α -, β -, and γ -asarone in each sample, instead of the absolute values (Table 2).

Principal Component Analysis (PCA) has revealed that the studied dataset was only two-dimensional, since the first two principal components extracted almost 100% (~99.99998%) information of the original data matrix. It was revealed that the relative concentration of α -asarone was almost perfectly, yet reversely correlated to the relative concentration of β -asarone, which again confirmed that both compounds have common origin and possibly could be transformed from one to another via enzymatic or light-induced E/Z isomerization. Hence, the information on α - and β -asarone constituted the first dimension of the examined dataset after applied VARIMAX rotation algorithm. The second dimension of the data contained solely information on the relative concentration of γ -asarone, which displayed no correlations to its α - and β - counterparts (Fig. 4).

Figure 4 presents a linear map of samples in the space of the two dimensions established above. Fresh samples collected from northern Poland (S1 & S2) exhibited lower than average relative concentrations of γ -asarone with and medium relative concentrations of α - and β -asarone. The S3 sample, collected from central Poland presented much lower relative amounts of γ -asarone in comparison to the rest of the sources. Samples S4 and S5, coming from commercially available rhizomes, displayed average relative concentrations of all three traced asarones. In contrary, sample S6, while maintaining relative amounts of γ -asarone similar to the ones of S5 and S6, exhibited highest—by far—relative concentration of α -asarone and, therefore, lowest relative concentration of β -asarone. Finally, the commercially available essential oils (S7–S9) presented the highest relative amounts of β - and γ -asarones and the lowest relative amounts of α -asarone in the whole studied group.

Cluster dendrogram (Fig. 5), prepared on the basis of the relative concentrations of all three studied asarones, has furtherly proved that extracts obtained from collected and commercially available rhizomes might be considered similar, with sample S6 being a potential outlier in the group—as PCA has proven, due to significantly different α/β ratio in comparison to samples S1–S5. Nevertheless, in comparison to extracts S1–S6, samples S7–S9, coming from commercially essential oils, exhibited fundamentally different asarone compositions, hence formed a separate cluster on the diagram.

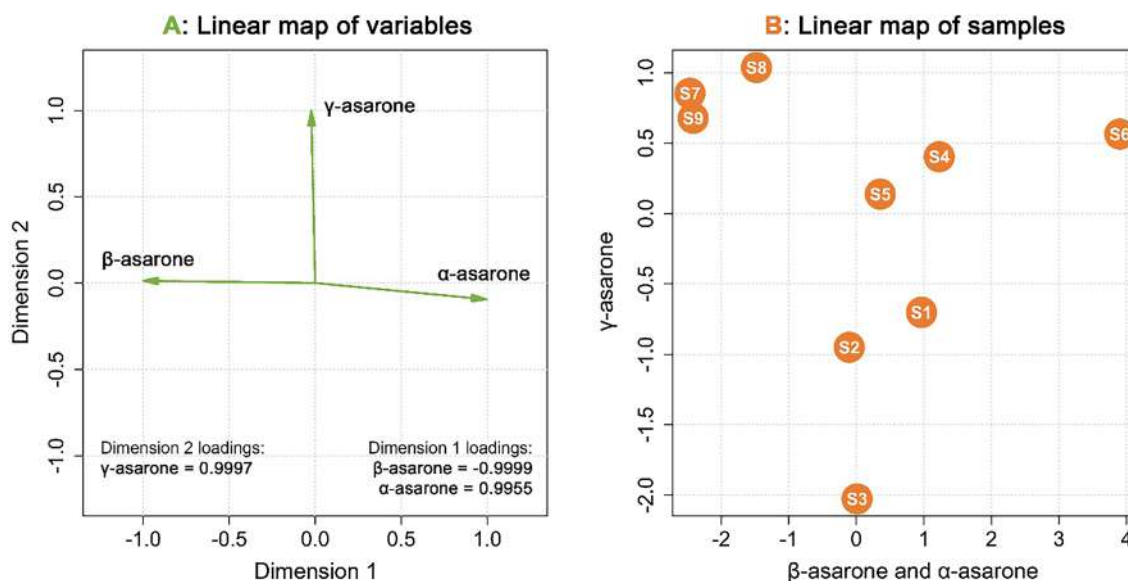


Figure 4. The linear map of original variables in the space of the two relevant dimensions of the discussed problem (A) and the linear map of samples in the same coordinate system (B).

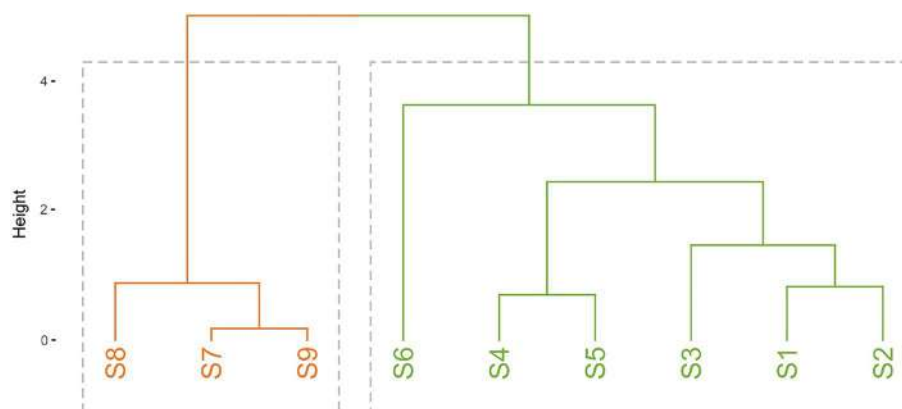


Figure 5. Cluster dendrogram, drawn on the basis of the Euclidean distances between studied samples, concerning relative concentrations of α-, β- and γ-asarone.

CPC method of removing β-asarone from *A. calamus* EO. Centrifugal partition chromatography (CPC) as an emerging purification technique is used in the separation process of natural products, protein mixtures, synthetic drugs, APIs and others. It belongs to green techniques due to a limited usage of solvents in the separation process, but most interestingly, it is more commonly selected by the industrial players as a technique reaching industrial-scale solutions⁴¹.

CPC chromatographs are used for industrial concentration and purification of several groups of natural products or single compounds, e.g. omega-3 fatty acids⁴² or acidic cannabinoids from *Cannabis sativa*⁴³. High selectivity and repeatability of the CPC-based analyses is certainly a strong advantage of this technique, however, its good operation must be based on a proper selection of the biphasic solvent system that should harmonize with the analysed sample and be selective towards the single constituents of the mixture.

Solvent system selection. The selection of the appropriate biphasic system plays a key role in counter-current chromatography, and therefore, this step should commence any method development of the separation process. Herein, an appropriate solvent system was chosen according to the partition and separation factors (K_d and α). Due to the hydrophobicity of target compounds, a series of low-polar solvent systems composed of n-hexane, ethyl acetate, methanol and water in different volume ratios (HEMWat/ Arizona system) were tested to provide an efficient separation of the target compounds. The Arizona system was also selected as there is no need for preparing various biphasic solvent systems in separating funnels (Gilson Glider software combined with a 4-channel pump is able to mix relative portions of the selected solvents directly in the apparatus). This aspect is important in case of pilot and industrial-scale application of developed method as it lowers the consumption of

System	n-Hex/EtOAc/MeOH/H ₂ O solvent system (v/v/v/v)	K _d		α
		α -asarone	β -asarone	
1	1:1:1:1	0.15	0.28	1.87
2	6:1:6:1	1.34	1.26	1.06
3	9:1:9:1	1.94	1.70	1.14
4	19:1:19:1	2.48	2.12	1.17

Table 3. Partition coefficient values calculated for some biphasic solvent systems and related selectivity for α -asarone and β -asarone. Selected solvent system is in bold.

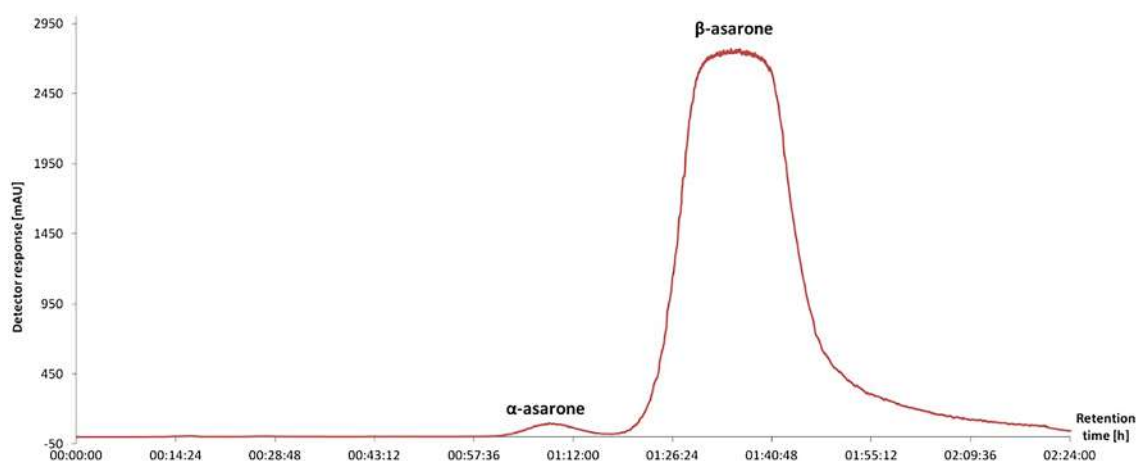


Figure 6. The CPC chromatogram (CPC-DAD) of the essential oil obtained from the rhizomes of *Acorus calamus* by hydrodistillation in the Clevenger apparatus. Monitored wavelength, $\lambda = 254$ nm.

solvents necessary for the separation process. Table 3 summarizes distribution coefficients (K_d) and separation factors (α) calculated for target compounds in the different volume ratios of HEMWat system.

Two of the tested two-phase solvent systems (system 2 and 3) met the first criterion of efficient separation with K_d in the range of 0.5–2⁴⁴. Among the two remaining systems, system 3 and 4 were selected for further evaluation on CPC chromatograph based on their beneficial differences between K_d values of the analysed components.

CPC separation. First, the experiment was run at different rotation speed of the column that was within a range of 1000–2300 rpm, at a constant flow rate of 5 mL/min to evaluate the impact of rotation speed on the selectivity. Finally 2200 rpm setting was selected as the most advantageous one, giving the best selectivity towards asarones with no drastic increase in the operating pressure. The selected relatively high rotation speed kept together with the flow rate of 5 mL/min generated the pressure of 50 bar that is half of the maximum operational pressure in CPC chromatographs. Sustaining a quicker flow rate as 5 mL/min markedly reduced the analysis time. During the optimisation time system No 4 was rejected, as it did not bring better selectivity towards both asarones, but only prolonged the analysis time of 20 min.

As a result of the CPC-based fractionation of *Acorus calamus* EO using the solvent system composed of n-Hex/EtOAc/MeOH/H₂O (9:1:9:1 v/v/v/v), the complete separation of both α - and β -asarones from the essential oil was obtained in a short time of 144 min in the elution mode (see Fig. 6 for the chromatogram from CPC chromatograph). The calculated Sf value of 72% is consistent with the stationary phase retention suggested for counter-current separations⁴⁵. It should be mentioned, that for all CPC columns, the Sf maximum value is necessarily much lower than 100% with the maximum value reaching 80%. Such values are dictated by the construction of the hydrostatic apparatus⁴⁶.

The fractions containing asarones were separated, whereas all remaining fractions were collected together. After the completion of the fractionation process the purity check of the isolates was performed using the HPLC-DAD instrumentation, compared with the total EO sample (see Fig. 7) and the purity of asarones was calculated as 93.7% for α - and 95.5% for β -asarone (see Table S4 in the Supplementary File). Interestingly, both compounds were eluted from the column in the elution mode, so as the remaining constituents of the essential oil could be easily pushed out from the column with a higher flow rate of 30 mL/min. This way, the performed fractionation of the EO succeeded in the separation of asarones from the remaining constituents of the oil. Collecting together all fractions except those containing asarones provides EO with no presence of asarones bearing toxic properties.

The elaborated purification protocol is the first one dedicated to the hydrostatic counter-current instrumentation, like CPC chromatographs. Previously, successful trials to recover asarones were performed on a different species, namely on *Acorus tatarinowii* EO and were published by Wang and co-investigators. The researchers

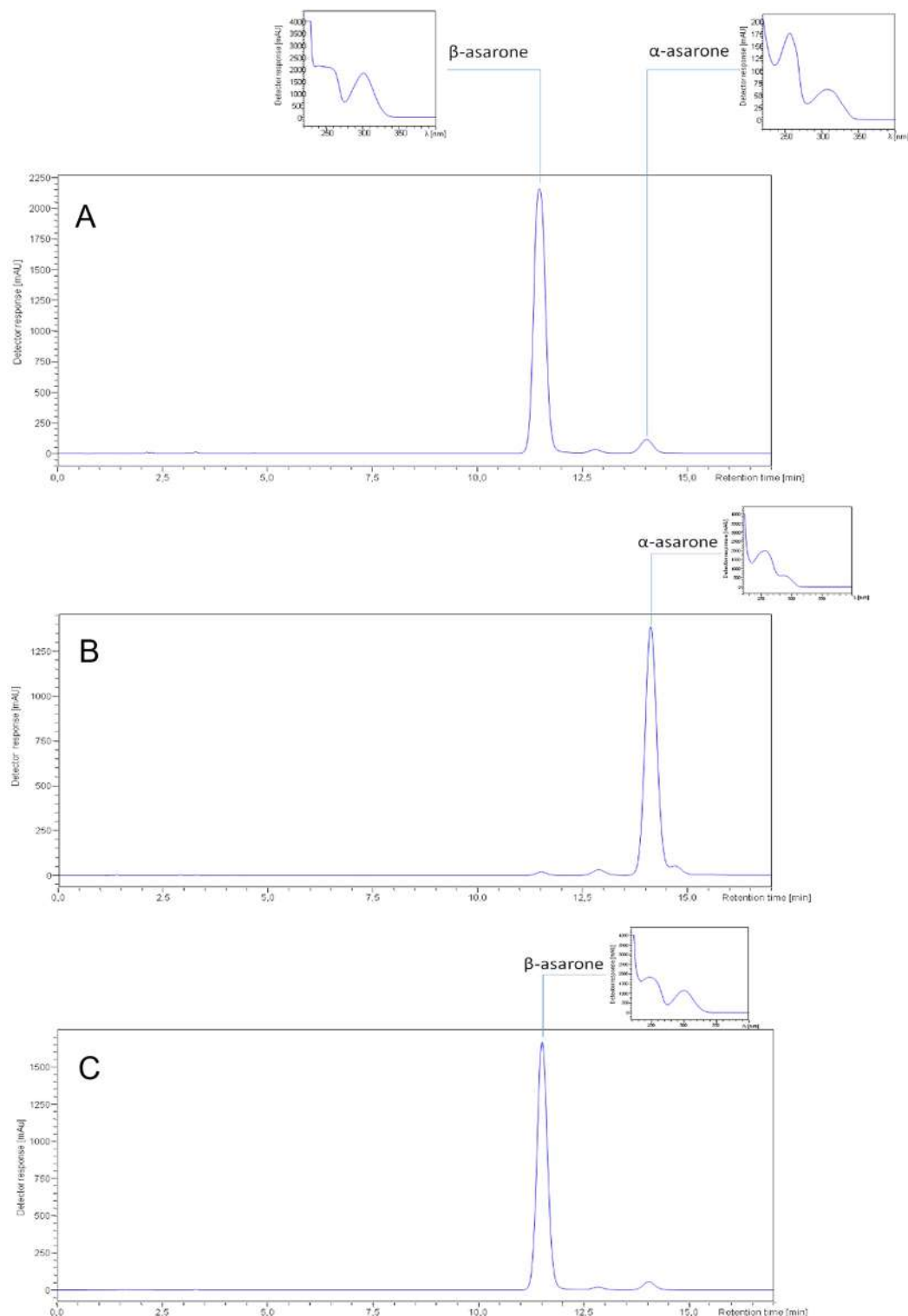


Figure 7. (A) HPLC-DAD chromatogram of the standard solution of *Acorus calamus* EO and UV spectra of α - and β -asarone; (B) HPLC-DAD chromatogram and UV spectrum of the α -asarone fraction obtained by CPC separation; (C) HPLC-DAD chromatogram and UV spectrum of the β -asarone fraction obtained by CPC separation.

used a different type of chromatograph in their study—an HSCCC chromatograph that is operated in the hydrodynamic mode. The used mixture of n-hexane–ethyl acetate–methanol–water (1:0.2:1:0.3, v/v) fractionated 1.0 g

Source	Rhizomes of natural accessions			Commercially available rhizomes			Commercially available EOs		
Code	S1	S2	S3	S4	S5	S6	S7	S8	S9
Country of origin	Poland	Poland	Poland	Hungary	Poland	Poland	Poland	Poland	India

Table 4. Samples of EOs obtained from natural and commercial resources.

Code	Origin (place/region)	Elevation (m amsl)	Latitude (North)	Longitude (East)	Harvesting time
S1	Filipów/Mazury Lake District	180	54°11'36"	22°39'26"	November 2021
S2	Gdańsk/Pomeranian Lake District	140	54°25'11"	18°26'50"	November 2021
S3	Małczew/Greater Poland lowland	230	51°46'40"	19°42'34"	November 2021

Table 5. The origin and harvesting time of the samples obtained from the natural accessions.

of the essential oil. The analysis was conducted for 6 h to obtain α - and β -asarones in the eluate. The authors used 800 rpm rotation speed of the column and the flow rate of only 1.5 mL/min. The selected parameters had impact on an increased purification time and small sample loading. Even if the previously described purification protocol provided high purity asarones, the application of HSCCC chromatographs is limited to analytical and semi-preparative conditions due to high pressure that is generated during the run.

Asarone-free calamus oil still shows potential as an eco-friendly green biopesticide or as a bioherbicide against weeds as it contains a mixture of many biologically active chemicals. Identified substances such as calarene, shyobunone, safrol, cedrene were detected also in other EOs exhibiting bactericidal, fungicidal, insecticidal, repellent or allelopathic activities, inhibiting germination different species^{47–51}. According to Liu and co-investigators⁵² α -asarone, methyleugenol and (*E*)-methylisoeugenol isolated from calamus oil were the strongest fumigants/insecticides and were found toxic against booklouse *Liposcelis bostrychophila*. In another study Chen et al.⁴⁷ underlined a strong impact of shyobunone and isoshyobunone isolated from calamus oil on the development of *Lasioderma serricorne* (LS) and *Tribolium castaneum* (TC). The isolates were characterized by a strong contact toxicity against LS and TC. The calculated LD50 values against LS adults were 20.24 and 24.19 μ g/adult specimen, respectively. The oil and the isolates were found to be strongly repellent already after 2 h of treatment.

The above examples show marked importance of calamus oil other than β -asarone. Based on this observation, the removal of this toxic component from the EO is logic and important especially these days, when new sources of repellent compounds that are neutral to environment are needed. Prior to extensive production, properties of the oil deprived of β -asarone, parallelly with the one containing β -asarone, must be thoroughly evaluated. Their impact on human cell lines, organisms present in the agrocenosis as well as germination and seedling development of various species should be analysed.

Conclusions

Chromatographic techniques are important tools suitable for the evaluation of plant extracts' composition. They are capable of fingerprinting⁵³ the extracts and also isolating single components from the matrix⁵⁴. Also in this study the qualitative analysis of the tested EO samples performed by HPLC and HPLC–MS led to the identification of 22 metabolites including β -asarone as a major constituent. According to our study, the content of β -asarone in the European origin EO depends on the location and can resemble the high content of the compound observed in tetraploid specimen from Asia. Having the widespread use of calamus oil in traditional medicine on the one hand, and the data on the toxicity of β -asarone on the other, it is very important to implement a procedure of β -asarone elimination from plant material. The proposed herein technique of removing β -asarone from the rhizome oil of *Acorus calamus*—the CPC chromatography—gives hope for the potential use of calamus oil as a potential bioherbicide—that could be safe for the environment and humans. Previously published data indicate strong repellent activity of other ingredients than β -asarone present in calamus oil, so its removal will not affect the total biocidal effectiveness. The CPC method proposed in this paper is simple, quick, meets the requirements of green chemistry practice, due to the relative low solvent consumption and promises simple upscaling conditions that would provide high quantities of β -asarone free calamus oil.

Methods

Plant material and extraction procedure. For this study, several samples of *Acorus calamus* rhizomes—both commercially available and collected from natural stands (see Table 4 and Table 5) were analysed. The material from personal collection came from different regions in Poland (see Fig. 8/Table 5) and was authenticated by prof. Justyna Polit from the Faculty of Biology and Environmental Protection of the University of Łódź in Poland. Also, commercially available calamus oil samples were used for the study. The commercially available *A. calamus* rhizomes and oil were obtained from the local herbal shop and came from different producers. The collection of the plant material and related studies complies with relevant institutional, national, and international guidelines and legislation.

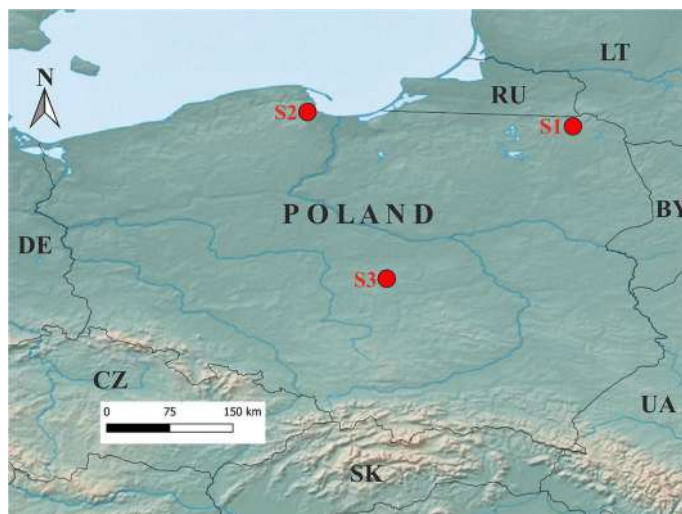


Figure 8. Map showing locations and origins of *Acorus calamus* accessions harvested from different regions of Poland. (S1–S3)—sample code, N—North, BY—Belarus, CZ—Czech Republic, DE—Germany, RU—Russian Federation, SK—Slovakia, UA—Ukraine⁵⁵. The base map of Poland and the neighbouring countries was obtained from <http://diva-gis.org/data>.

First, the personally collected rhizomes were finely cut and dried in the air in the shade. The obtained plant material and the purchased samples of dried rhizomes were subjected to hydrodistillation. Every time fifty grams of plant material were covered with 100 mL of water, sonicated for 15 min at room temperature and subjected to hydrodistillation in Deryng apparatus for 4 h. The obtained essential oil was later collected from the apparatus to a glass vial, dried from water using sodium sulfate, and diluted 100 times with methanol. The extracts were filtered through a nylon syringe filter (pore diameter of 0.22 μ m, Sigma Aldrich, St. Louis, MO, USA) and immediately subjected to compositional studies. The purchased essential oil samples were diluted 100 times prior the compositional analysis, filtered and analysed as described above.

Identification of components in the EO by HPLC-ESI-QTOF-MS/MS. Qualitative analysis of the analysed samples and fractions from CPC analyses was performed by an analytical platform—the HPLC-ESI-QTOF-MS/MS chromatograph 6500 Series (Agilent Technologies, Santa Clara, CA, USA) composed of a binary pump, a degasser, an autosampler, a thermostat, a DAD detector and mass spectrometer (G6530B). The following chromatographic conditions were applied in the study: flow rate of 0.2 mL/min, temperature of 21 °C, injection volume of 2 μ L, the UV signals: 280, 254, 210, 230 and 320 nm and the run time of 55 min. The gradient of acetonitrile (solvent B) and water—both with the addition of 0.1% formic acid were used in the following program: 0 min—1% B, 4 min—30% B, 34 min—60% B, 40 min—95% B, 44 min 95% B, 45—1% B. The chromatographic separation was performed on a Zorbax Eclipse Plus RP-18 column (150 mm $\times$ 2.1 mm; dp = 3.5 μ m) produced by Agilent Technologies (Santa Clara, Ca, USA).

Freshly calibrated and tuned mass spectrometer was operated within the m/z range of 40–1200 u, in the further described settings: gas temperature of 250 °C, sheath gas temperature of 300 °C, gas and sheath gas flows of 12 L/min, nebulizer pressure of 35 psig, capillary voltage of 2500 V, nozzle voltage of 1000 V, fragmentor voltage of 100 V, collision energies of 10 and 20 V, skimmer voltage of 65 V. Mass Hunter Workstation Software (v. B.08.00) was used to record and process the collected data. The structure elucidation was based on the comparison of the obtained data with those present in the scientific literature (retention time, high accuracy mass measurement, the analysis of their MS/MS spectra) in a constructed method that aimed at the fragmentation of two the most intensive m/z signals in each scan, in two collision energies.

Quantitative analysis of β -asarone content in EO by HPLC-DAD. The quantitative analysis of asarones in EO samples was performed by HPLC-DAD chromatograph produced by Shimadzu (Kyoto, Japan). The instrument was composed of a quaternary pump (LC-20AD), a degasser (DGU-20A), an autosampler (SIL-20A HT), a Photodiode Array Detector (SPD-M20A), and a column oven (CTO-10AS VP). The collection and handling of data was supported by the LabSolutions program. The chromatographic separation was performed on Phenomenex Luna 100 C18 (2) column (150 $\times$ 4.6 mm, 5 μ m) (Phenomenex, Torrance, CA, USA). The isocratic elution mode of 55% methanol in water was applied (v/v). The flow rate was set at 1 mL/min and the detection wavelength used to calculate the content of asarones was selected as 235 nm. First, the concentration of β -asarone in the tested samples was calculated based on the calibration curve of the authentic standard. For this purpose a freshly prepared 2 mg/mL solution of β -asarone standard in methanol (95% HPLC purity, Sigma Aldrich, St. Louis, MO, USA) was injected in the concentration range of 0.01–2 mg/mL from 5 individual solutions prepared by the dilution of the stock. The obtained calibration curve equation was calculated as $y = 160320853x - 5933.5$

and the regression coefficient value as $R^2 = 0.9999$. Also, the relative amount of asarones (α -, β - and γ -asarone) was calculated based on the HPLC chromatograms.

Statistical analysis. All the chemometric analyses and visualizations were performed using R v4.2.0⁵⁶ programming language in RStudio⁵⁷ software with pracma⁵⁸, factoextra⁵⁹, and matlib⁶⁰ packages installed.

Principal component analysis (PCA). Before the assessments, all peak areas of the three traced asarones of all nine samples were summed to 100%, hence the amount of every asarone was represented as a percentage share of a total detector response of a given source (Table 2). After the percentage rescaling of samples, all the variables (i.e. α -, β - and γ -asarone) were autoscaled in order to enable a formal decomposition of the covariance matrix. As a result of the application of standard principal component analysis (PCA) algorithm, first two principal components (PCs) were considered relevant in the presented case. In the next step, their vectors were rotated in space in order to maximize values of correlation coefficients between the original variables and the two orthogonal factors using VARIMAX approach. The linear map of samples (sources) in the space of the resulting varivectors (referred to as 'dimensions' in the main text) was prepared by multiplying the matrix of original variables' loadings in the space of varivectors by the matrix of the autoscaled dataset (see Tables S2 and S3 in the Supplementary File).

Hierarchical clustering. Hierarchical clustering for the studied asarones' case was executed using Ward method, basing on a standard matrix of Euclidean distances between the sources.

Centrifugal partition chromatography method of removing β -asarone. *Biphasic solvent system selection.* The biphasic solvent system to be used for the fractionation of *Acorus calamus* EO by means of Centrifugal Partition Chromatography was established by determining its partition coefficient (K_d) in several biphasic solvents mixtures, using the shake-flask method. The selection criteria for the system were based on the differences in partition coefficient values of single constituents of the extract and the selectivity (α) of every system for α -asarone and β -asarone. These parameters were determined as follows: 4.95 mL of upper and lower phases of the corresponding solvent system were placed in a test tube and 0.1 mL of the *Acorus calamus* EO was added. The tubes were vigorously shaken and after equilibrium was achieved, both phases were separately filtered through a syringe filter (pore diameter of 0.22 μ m) into separate vials and analysed by HPLC-DAD using the chromatographic conditions described in section *Quantitative analysis of β -asarone content*.

The K_d values of α -asarone and β -asarone were defined as the peak area of the corresponding compound in the lower phase divided by the peak area of the compound in the upper phase. Next, K_d ratios of each compound were calculated to determine the selectivity (α) of each solvent system, according to Ito and Golden⁴⁴ and the formula below.

$$\alpha = K_{d1}/K_{d2} \quad (1)$$

where: K_{d1} , K_{d2} —partition coefficient of two asarones, where $K_{d1} > K_{d2}$.

***Acorus calamus* EO fractionation.** The removal of β -asarone from the essential oil was performed by means of centrifugal partition chromatography (CPC) on a Gilson CPC-250 apparatus (Gilson Inc., Middleton, WI, USA), equipped with the Ecom flash 14 DAD 600 detector (Gilson Inc., Middleton, WI, USA) and Gilson fraction collector LS-5600 (Gilson Inc., Middleton, WI, USA). The biphasic liquid system HEMWat/ Arizona X (n-hexane/EtOAc/MeOH/water, 9: 1: 9: 1 v/v/v/v) was prepared by mixing the respective portions of the solvents at room temperature. The separation was performed in ascending mode, using the upper phase as the mobile phase. First, the lower phase was loaded to the column at a flow rate of 30 mL/min, at rotational speed of 500 rpm and the time of 12 min. Next the upper phase was pumped through the stationary phase for the following 13 min at a flow rate of 5 mL/min, rotational speed of 2200 rpm, until equilibrium was achieved with a pressure of 50 bar. The stationary phase retention (S_f) was determined according to Berthod and Faure⁴⁵ and the equation below.

$$S_f = V_s/V_c \quad (2)$$

where: V_s —retained volume of stationary phase in the column after the equilibrium; V_c —column volume.

Then, 1 mL of the *Acorus calamus* essential oil, dissolved in 9 mL of equal volumes of upper and lower phase (1:1, v/v), was introduced via manual injection valve and an injection loop of 10 mL. The upper phase was pumped at the analytical conditions during 150 min, while fractionation occurred (12 mL/fraction). Afterwards, the lower phase was introduced to the column at a flow rate of 30 mL/min and a rotational speed of 500 rpm during 10 min, as an extrusion step, totalizing 160 min of the fractionation. The effluent was monitored at a wavelength of 254 nm into 12 mL volume vessels. Data were collected and processed with Gilson Glider CPC V5.1d.01 software. The composition of fractions was confirmed by HPLC-ESI-QTOF-MS/MS instrumentation, whereas their purity with help of HPLC-DAD apparatus according to the previously described protocol. All the fractions except those corresponding to asarones were combined and the solvents evaporated under reduced pressure at the temperature not exceeding 40 °C.

Data availability

Most of the data generated or analyzed during this study are included in this published article (and its Supplementary Data files).

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Author contributions

P.S., J.T.P., L.S. and W.K.-K. conceived the experiments, M.W., T.B., J.G., M.K. and J.B.-B. conducted the experiments, P.S., T.L., J.T.P. and W.K.-K. analyzed the results. P.S., M.W., J.T.P., T.L. and W.K.-K. wrote the main manuscript and prepared Figs. 1, 3, 4, 5, 6, 7 and 8 and A.S. prepared Fig. 2. P.S., W.K.-K., M.W. and A.S. prepared revised version of the manuscript. All authors have reviewed the manuscript.

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Competing interests

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Additional information

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Research Paper

Phytotoxicity and bioherbicidal potential of sweet flag (*Acorus calamus* L.) essential oil on Fabaceae and Brassicaceae species

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ABSTRACT

Given the growing need to phase out chemical pesticides, plant-derived compounds are being investigated as eco-friendly alternatives. Sweet flag (*Acorus calamus*) essential oil (SEO), traditionally used in Chinese medicine, remains underexplored in terms of its phytotoxic properties and agricultural potential. This study evaluated the phytotoxic effects of 24-hour seedling incubation with emulsified SEO on primary root growth, cell viability, oxidative stress, overall metabolism (protein and sugar levels, fatty acid profile), and lipid peroxidation in Fabaceae (*Vicia faba*, *Lupinus luteus*) and Brassicaceae (*Brassica napus*, *Arabidopsis thaliana*), focusing on inter-family and interspecies selectivity. Half-maximal inhibitory concentrations (IC₅₀) were determined as follows: 0.03 % for *V. faba*, 0.025 % for *L. luteus*, 0.01 % for *B. napus*, and 0.005 % for *A. thaliana*. Despite similar stress intensities defined by IC₅₀, SEO reduced root biomass in a species-dependent manner. Oxidative stress was confirmed by increased reactive oxygen species (ROS), with the highest H₂O₂ accumulation observed in Fabaceae, reaching 267 % of control in *V. faba* and 340 % in *L. luteus*, but staying below 200 % in Brassicaceae. Nonetheless, Fabaceae activated more efficient antioxidant defenses, as reflected by species-specific modulation of ascorbate, glutathione, carotenoids, and the activities of SOD, APX, GR, and CAT. In contrast, Brassicaceae experienced stronger metabolic disturbances, evidenced by enhanced heat emission (isothermal calorimetry), increased lipid peroxidation, membrane damage, elevated electrolyte leakage, and a greater decline in cell viability. These results underscore the species-specific responses to SEO and highlight its selective phytotoxicity, supporting its potential as a sustainable bioherbicide that effectively targets Brassicaceae weeds while preserving Fabaceae crops.

1. Introduction

Chemical pesticides have been widely used for plant protection against weeds, fungi, and herbivores since the 20th century. While their primary benefit lies in ensuring food security, these chemical agents can lead to the development of resistance in pests and weeds, and they are toxic to humans and non-target organisms through direct exposure during agricultural operations (Assadpour et al., 2024; Dehnert et al., 2019; Nakka et al., 2019; Perrin et al., 2021). Residues remaining in

food may also adversely affect consumer health. Owing to their high chemical stability, these compounds are poorly degradable, leading to their accumulation in soil and contamination of groundwater—a process further intensified by the continued application of increasing doses (Silva et al., 2019). The global challenge of rising herbicide resistance among weeds and environmental degradation caused by chemical herbicides underscores the urgent need for alternative weed management strategies. To minimize the damages, the European Union implemented a strategy, that assumes, among others, a reduction in pesticide use by

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50 % by 2030 and the development of sustainable, biologically originated crop protection products (Schebesta and Candel, 2020). This really ambition initiative underscores the importance of the comprehensive studies about bio-pesticides, their toxicity, and mechanisms of action which, in the light of the upcoming 2030 goals, are still poorly understood.

One promising solution, in the area of searching for new bio-pesticides, lies in plant secondary metabolism which produces, among other things, a variety of essential oils. These volatile compounds are synthesized by all plant organs and are responsible for the scent and taste of plants. Nevertheless, they also play semiochemical functions by attracting pollinators and repelling herbivorous insects, and allelopathic functions, have an inhibitory or stimulatory effect on nearby plants. Aside from that, acting as signaling molecules, they play a role in plant adaptation to biotic and abiotic stresses and plant-plant communication (Araniti et al., 2013b; Assadpour et al., 2024; Dhifi et al., 2016; Pavela and Benelli, 2016; Raveau et al., 2020; Sharifi-Rad et al., 2017; Synowiec and Drozdek, 2016; Usano-Aleman and Herraiz-Peñalver, 2016; Verdeguer et al., 2020).

Essential oils are complex mixtures of chemical compounds possessing a variety of biological activities, making them promising tools in many applications (Assadpour et al., 2024). Due to scent, antioxidative, antiseptic, and anti-inflammatory properties, essential oils are valued in food, cosmetics, textile, pharmaceutical, and medical industries (Abd-ElGawad et al., 2019). Moreover, their antimicrobial, antifungal, and insecticidal properties enable them to control pathogens and pests (Pavela and Benelli, 2016). Additionally, they demonstrate a strong potential as bioherbicides facilitating crop protection against weeds (Assadpour et al., 2024; Foncoux et al., 2023; Gupta et al., 2023; Islam et al., 2024; Raveau et al., 2020; Verdeguer et al., 2020). Typically, essential oil consists of 20–60 active substances whose composition depends on plant species, climatic conditions, the presence of endophytes, or even on plant ploidy (Raveau et al., 2020; Uebel et al., 2021). For the time being, around 3000 essential oils have been identified which led to classify their major components, responsible for biological activity, into terpene hydrocarbons (monoterpenes, sesquiterpenes) and oxygenated compounds (alcohols, phenols, aldehydes, and esters) (Verdeguer et al., 2020).

Due to the chemical variety and complexity of essential oil mixtures, their biological activity is primarily determined by the presence of minor compounds that act synergistically with the minor ones and modulate their activity which is the key to preventing the target organisms from acquiring resistance (Araniti et al., 2013a; Synowiec et al., 2019). Moreover, being susceptible to degradation, they cannot be considered as toxic substances (Assadpour et al., 2024; Raveau et al., 2020).

Most essential oils trigger responses to abiotic stress in plants through the overproduction of ROS (Werrie et al., 2020). Oxidative stress is therefore a common secondary stress accompanying external stress factors. Physiologically produced ROS are involved in developmental processes and act as signaling molecules (Huang et al., 2019; Werrie et al., 2020). However, excessive amounts of ROS generated during the plant stress response to essential oils are highly toxic and activate a cascade of ROS-scavenging reactions involving both enzymatic and non-enzymatic components (Dumanović et al., 2021; Kaur et al., 2012; Wang et al., 2024). If the protective activity of antioxidant systems toward cellular structures and processes is insufficient, damage may occur to cell membranes, organelles, genetic material, as well as structural and enzymatic proteins. As a consequence, processes such as replication, transcription, translation, respiration, and photosynthesis become disrupted, ultimately leading to the death of the cell, tissue, or the entire organism (Depaepe et al., 2021).

An essential oil displaying curious properties is extracted from the rhizomes of sweet flag (*Acorus calamus* L.), a plant used in traditional Chinese medicine. There are numerous studies evaluating a variety of its medical properties improving mental and physical health (Bai et al.,

2022; Das et al., 2019; Mukherjee et al., 2007; O'Neill et al., 2017; Rajput et al., 2014). The compounds present in the sweet flag essential oil (SEO) include monoterpenes, sesquiterpenes, and the most abundant phenylpropanoids such as α -, β -, and γ -asarones whose content is determined by plant ploidy (Ogra et al., 2009; Uebel et al., 2021). The identification of SEO components has been previously performed (Bai et al., 2022; Madhuri et al., 2024; Szczeblewski et al., 2022). It follows that the biological activity of the tested oil could be attributed mainly to the presence of asarones (Madhuri et al., 2024; Sharma et al., 2020; Szczeblewski et al., 2022) which are the main and most important SEO components (Satyal et al., 2013) found in *Asarum* sp. plants as well (Liu and Wang, 2022). Asarones due to their ability to interact with cellular redox status can act dually: prooxidative and antioxidative. In, regarded as toxic, high concentrations (above 0.5 % v/v) these compounds can induce reactive oxygen species (ROS) production leading to lipid peroxidation, cell membrane damage, and function loss. In lower ones (0.01–0.1 % v/v), they can act as protectants by scavenging ROS, preventing from initiation or propagation of lipid peroxidation. Moreover, they activate signaling pathways and enhance the activity of transcription factors critical for antioxidant enzymes' induction (Lam et al., 2017). These data, however, relate to the animal (Saki et al., 2020) or *in vitro* studies conducted on animal cell lines (astrocytes, fibroblasts, hepatocytes, and cancer cells) (Lam et al., 2017). Unfortunately, the literature lacks data explaining the mechanisms of action of SEO or pure asarones in plant cells. Only a few studies are available, and they are limited to evaluating the germination and seedling growth of *Lactuca sativa* L. and *Lolium perenne* L. under the influence of SEO (Satyal et al., 2013), or to demonstrating the inhibitory effect of an aqueous extract from *A. calamus* rhizome on mitotic progression in *Allium cepa* L. roots (Rajamanikam et al., 2019).

The aim of project was to assess the phytotoxic potential of sweet flag (*Acorus calamus* L.) essential oil (SEO) during 24-hour incubation on primary root growth across two Fabaceae (*Vicia faba* var. *minor* L. and *Lupinus luteus* L.) and two Brassicaceae (*Brassica napus* L. and *Arabidopsis thaliana* L.) species and to determine for each of them the concentration required for half-maximal growth inhibition (IC₅₀). We hypothesized that SEO would act selectively, exhibiting bioherbicidal potential by enabling the control of Brassicaceae plants during Fabaceae cultivation.

Determining IC₅₀ values served two purposes: it revealed the sensitivity of each species to SEO and established a comparable stress level across species, corresponding to 50 % inhibition of primary root growth. This experimental model enabled analyses of SEO's mode of action by allowing the detection of subtle physiological and molecular responses under moderate stress conditions that do not lead to organismal death but instead trigger defense mechanisms and metabolic adjustments. It also made it possible to evaluate whether plant responses were uniform, species-specific, or showed trends characteristic of each family.

Thus, to determine the potential bioherbicidal properties of SEO and to gain insight into its mode of action in root cells, we analyzed its effects on tissue hydration and biomass synthesis capacity, measured through changes in fresh and dry weight and electrolyte leakage into the growth medium. We assessed cell viability, accumulation of reactive oxygen species (ROS) in the form of H₂O₂, and the performance of non-enzymatic and enzymatic antioxidant systems. The latter were evaluated by measuring the levels of ascorbate, glutathione, cysteine, γ -glutamylcysteine, and α - and β -carotene, as well as the activities of superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC:1.8.1.7), and catalase (CAT, EC 1.11.1.6). The overall metabolic activity of seedlings was also examined using isothermal calorimetry and changes in the content of proteins, monosaccharides, and saturated and unsaturated fatty acids were analyzed. The extent of cellular damage was assessed by lipid peroxidation, reflected by malondialdehyde (MDA) content.

The selection of species representing two plant families was based on their economic importance and research utility, enabled a comparative analysis of their sensitivity to SEO. Species from the Fabaceae family are

among the most economically significant crops, occupying the largest cultivation areas among grain legumes with large, protein-rich seeds. However, their slow growth at the beginning of vegetation favors the dynamic development of competitive weeds. In addition, the limited number of herbicides dedicated to these crops makes weed control in Fabaceae cultivation difficult (Pawłowski, 2018; [REPORT FROM THE COMMISSION TO THE COUNCIL AND THE EUROPEAN PARLIAMENT on the development of plant proteins in the European Union](#), 2018). On the other hand, *B. napus*, despite being a crop species, is an extremely troublesome and invasive volunteer plant for leguminous crops, difficult to eliminate in monoculture (Weber et al., 2014). Meanwhile, *A. thaliana* is simultaneously a weed, and a model plant, with a fully sequenced genome, used in molecular studies (Ferjani et al., 2023), which can be further developed in the next stages of research focusing on the effects of SEO. Moreover, in future cytological studies, *V. faba* var. *minor* will serve as an excellent model species due to its large, clearly visible chromosomes under the microscope. For immunocytochemical analyses, *L. luteus* and *B. napus*, characterized by low DNA content and a delicate, reticulate interphase chromatin structure, will be well suited to epigenetic investigations involving the binding of specific antibodies.

Therefore, this study not only evaluates the phytotoxic potential of sweet flag essential oil, but also establishes a model system for investigating the cellular and molecular mechanisms underlying its action in root tissues. In contrast to previous studies focused mainly on germination or mitotic inhibition (Rajamanikkam et al., 2019; Satyal et al., 2013) our approach integrates physiological, biochemical, and metabolic analyses across multiple species representing two economically important plant families. This provides a broader and more mechanistic perspective on plant responses to natural allelochemicals. The results are expected to contribute to the identification of species- or family-specific stress responses, enhance our understanding of plant tolerance mechanisms triggered by natural bioactive compounds, and support the development of selective, environmentally friendly weed management strategies.

2. Material and methods

2.1. Plant material

Seed material was obtained from the following sources: *DANKO Hodowla Roślin* Sp. z o.o. (Choryń, Poland) – faba bean (*Vicia faba* L. subsp. *minor*, cv. Bobas); *Poznańska Hodowla Roślin* Sp. z o.o. (Tulce, Poland) – yellow lupine (*Lupinus luteus* L., cv. Baryt); *Hodowla Roślin Strzelce* Sp. z o.o. (Strzelce, Poland) – rapeseed (*Brassica napus* L., cv. Markus); and the *Nottingham Arabidopsis Stock Centre* - NASC, (Nottingham, UK) – thale cress (*Arabidopsis thaliana* Col-0 (L.) Heynh.).

2.2. Seed germination

Seeds of four plant species were germinated in standard conditions: on double-layered filter paper soaked with deionized water in sterile Petri dishes. Due to differences in seed size, Petri dishes of varying diameters with different amounts of water were used: 9 cm (4 mL water) for *A. thaliana*, 11 cm (9 mL) for *B. napus*, 14 cm (15 mL) for *L. luteus*, and 17 cm (23 mL) for *V. faba*. The amount of water used ensured complete moistening of the filter paper without seed waterlogging. The dishes were kept in darkness at 23 °C for 2–3 days, with the exception of *A. thaliana*, which was germinated under long-day photoperiod conditions (16 h light / 8 h dark) due to its sensitivity to etiolation. After germination, seedlings with comparable primary root lengths (approximately 2 cm for *V. faba* and *L. luteus*, 1 cm for *B. napus*, and 0.5 cm for *A. thaliana*) were selected for essential oil treatment.

2.3. Essential oil treatment

Sweet flag essential oil (SEO; Etja, Poland) of known composition

was used for experiments (Szczęblewski et al., 2022). For proper SEO distribution in water, it was emulsified in the mixture of C14–18 and unsaturated C16–18 – mono- and di-ethoxylated glycerides and ethoxylated *B. napus* oil. The above-mentioned emulsifier was mixed with SEO in 1:4 (v/v) ratio and strongly shaken using a vortex for 15 min according to the procedure standardly used in greenhouse research (Grzanka et al., 2022). The emulsifier-to-essential-oil ratio was adopted based on previous trials, which indicated that this proportion ensured a stable and homogeneous emulsion. According to the preliminary studies, for seedlings incubation, emulsified SEO was dissolved in water to the range of concentrations (0.01 – 0.05 % for *V. faba*, *L. luteus*, *B. napus*, and 0.004 – 0.009 % for *A. thaliana*). Seedlings were incubated on double-layered filter paper in the presence of 15 ml of prepared solutions in tightly sealed (with Parafilm) 150 mm ø Petri dishes for 24 h. Seedlings incubated in water and dissolved emulsifier in the concentration used for SEO emulsification were considered as controls. Further analyses were performed in three or four experimental repeats of the control, emulsifier series (E), and SEO series (O).

2.4. Root length measurement, IC₅₀ determination, and fresh/dry weight analysis

Seedlings were photographed and measured using ImageJ ver. 1.54e before and after treatment from the root tip to the hypocotyl. Root length increments were calculated by subtracting the length of the root before treatment from the length of the root after treatment. To standardize plant responses for tested SEO and ensure the reproducibility of experiments we calculated IC₅₀ values for each tested species. This value represents a dose of the tested compound inhibiting root growth by 50 % crucial for determining a mechanism of action of the tested compounds and delineating differences and similarities within the tested species (Landi et al., 2020). Calculated values of root length increments were recalculated as a percentage of control and the averages from 3 experimental repeats were used to calculate in GraphPad Prism 10 software (Boston, MA, USA) the IC₅₀ concentration using non-linear regression. Seedlings incubated in determined IC₅₀ (the concentration that inhibits root length increment by 50 % for each species) were chosen for further analyses. IC₅₀ concentration of SEO was: 0.03 % - *V. faba*, 0.025 % - *L. luteus*, 0.01 % - *B. napus*, and 0.005 % - *A. thaliana*, respectively. Fresh (FW) and dry weight (DW) measurements of excised primary roots were performed in 3 biological replicates according to (Kązmierzak et al., 2022).

2.5. Cell viability measurements

The viability was determined using Evans blue assay according to (Guidotti et al., 2013) with a minor modification. Roots were incubated in 3 ml of 0.25 % Evans blue for 15 min and rinsed twice in distilled water for 15 min. each. Then, root tips were cut and incubated in 3 ml of *N*, *N*-Dimethylformamide (DMF) for 50 min at room temperature. The absorbance was measured at 600 nm. To achieve a positive control, roots were treated for 1 h in 10 % H₂O₂ solution before Evans blue treatment. Evaluation was performed for 4 biological replicates.

2.6. Conductivity measurements

Conductivity was measured using a conductivity meter (Elmetron, Poland) in 3 ml of the culture media after 24-h seedlings incubation in water, dissolved emulsifier in the concentration used for SEO emulsification and SEO in IC₅₀. Evaluation was performed for 3 biological replicates.

2.7. Determination of seedlings' metabolic activity

The metabolic activity of seedlings was determined using isothermal calorimetry according to (Saja et al., 2014) with minor modifications.

Seedlings of four tested species incubated in 1 ml of emulsifier and SEO were placed into measuring ampoules. The measurements were performed for 24 h at 23 °C using TAM III isothermal calorimeter (TA Instruments, New Castle, USA) after 30 min of instrument stabilization perturbed by inserting samples. The measurements were performed in one repetition using 2–3 seedlings for Fabaceae species, 20 seedlings for *B. napus*, and 10 seedlings for *A. thaliana*. The time of incubation, when the most intensive changes in heat emission for each species were noticed, was used for further experiments.

2.8. Determination of H₂O₂ and lipid peroxidation

Excised snap-frozen 100 mg of roots were homogenized in a bead mill, incubated in the presence of 5 % (w/v) trichloroacetic acid (TCA) for 10 min and centrifuged for 15 min (9000 × g, 3 °C). For H₂O₂ content determination, the supernatant was mixed in equal parts with a reaction mixture consisting of 2.5 mM potassium phosphate buffer (pH 7) and 500 mM potassium iodide. The absorbance was measured at 390 nm (Talarek-Karwel et al., 2020; Velikova et al., 2000). The evaluation was performed for 4 biological replicates.

The malondialdehyde (MDA) content was measured to determine a lipid peroxidation level. The supernatant was mixed with 0.5 % thiobarbituric acid (TBA) in 20 % TCA, and incubated at 95 °C for 20 min. Then, the reaction tubes were placed on ice, and centrifuged. Then, the absorbance was measured at 532 and 600 nm. The nonspecific absorption was subtracted from the absorbance measured at 532 nm, and the content of MDA-TBA was calculated using the extinction coefficient = 155 mM⁻¹cm⁻¹ (Cakmak and Horst, 1991; Heath and Packer, 1968; Talarék-Karwel et al., 2020). The evaluation was performed for 4 biological replicates.

2.9. Monosaccharide content evaluation

Excised roots were homogenized in 2 ml of ethanol at 75 °C in a bead mill and centrifuged (9000 × g) for 10 min. Samples were left to dry and the residue was resuspended in 1 ml of water and desalted using ion-exchange resin (Amberlite MB3). After mixing the samples with 50 µl 0.1 M potassium ferricyanide, 100 µl of reagent A [0.1 M NaOH, 0.1 N Na₂CO₃; 1:1 (v/v)] was added and filled up with water to 1000 µl. Then, the solutions were heated for 10 min at 95 °C and cooled down to 20 °C. 1 ml of reagent B [0.015 M o-phenanthroline, 0.1 M acetic acid; 1:1 (v/v)] and 0.5 ml of water were added and heated for 10 min at 95 °C being simultaneously mixed. The absorbance was measured after cooling down the samples to 20 °C at 505 nm (Somogyi, 1952; Talarék-Karwel et al., 2020). The evaluation was performed for 4 biological replicates.

2.10. Protein content evaluation

100 mg excised roots were homogenized in a bead mill in extraction buffer consisting of 50 mM phosphate buffer (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride, 0.5 % (v/v) Triton X-100, and 2 % (w/v) polyvinylpyrrolidone (PVP)-30 and centrifuged for 15 min (9000 × g, 3 °C) (Talarék-Karwel et al., 2020). The protein content was evaluated using the Bradford method with bovine serum albumin as a standard (Bradford, 1976). 100 µl was mixed with 3 ml of the Bradford reagent consisting of 0.01 % (w/v) Coomassie Brilliant Blue G-250, 4.7 % (w/v) ethanol, and 8.5 % (w/v) H₃PO₄. The absorbance at 595 nm was measured after 5 min and, to check the protein-dye complex stability, before 1 h. The evaluation was performed for 4 biological replicates.

2.11. Antioxidant enzyme activity determination

Delineating the activity of ascorbate peroxidase (APX) was performed according to (Nakano and Asada, 1981) by detecting the absorbance rate at 290 nm using the extinction coefficient 2.8 mM⁻¹

cm⁻¹. The designation was performed in 3 ml of reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7), 0.5 mM ascorbate, 1 mM H₂O₂, and 100 µl of enzyme extract in 3 min. 25 °C. The enzyme activity was determined as the enzyme content that oxidizes 1 µM of ascorbate per milligram of soluble protein per minute.

Superoxide dismutase activity (SOD) was evaluated by the detection of photochemical reduction inhibition of neotetrazolium blue (NBT) at 560 nm (Beauchamp and Fridovich, 1971). 3 ml reaction mixture consisted of 50 mM sodium carbonate (pH 10.2), 24 µM NBT, 0.1 mM EDTA, 1 µM hydroxylamine, 0.03 % (v/v) Triton X-100, and 70 µl enzyme extract. The quantity required to achieve a 50 % reduction in phytochemical-mediated NBT reduction was considered equivalent to one unit of SOD per milligram of protein.

Catalase activity (CAT) was determined via (Aebi, 1984). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 15 mM H₂O₂, and 100 µl enzyme extract. The absorbance rate was measured in a 3 ml reaction mixture at 240 nm for 0.5 min at 25 °C. One unit of enzyme activity was defined as the amount of enzyme decomposing 1 µM of H₂O₂ per milligram of soluble protein per minute.

Glutathione reductase (GR) activity measurements were performed according to (Schaedle and Bassham, 1977) in the volume of 3 ml. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.6), 1 mM oxidized glutathione, 0.5 mM EDTA, and 0.1 mM reduced NADPH mixed with 100 µl of enzyme extract. Then the reaction was launched using 0.1 mM NADPH at 25 °C. The measured rate of NADPH oxidation resulting from the decrease of absorbance at 340 nm was used to calculate the GR activity using the extinction coefficient equal to 6.2 mM⁻¹cm⁻¹.

Evaluation of the enzyme activity was performed for 4 biological replicates.

2.12. Determination of ascorbate, cysteine, γ-glutamylcysteine, and glutathione content

Ascorbate (ASC) content was determined according to Kampfenkel et al. (Kampfenkel et al., 1995) method. Plant tissue was homogenized in 2 ml of 5 % (w/v) TCA in a bead mill for 5 min and centrifuged for 10 min (9000 × g). The supernatant was mixed with the reaction mixture consisting of 10 mM DTT, 0.2 M phosphate buffer (pH 7.4), 0.5 % N-ethylmaleimide, 10 % TCA, 42 % H₃PO₄, 4 % 2,2'-bipyridyl and 3 % FeCl₃ and then incubated for 40 min. at 42 °C. The absorbance was measured at 525 nm. Evaluation was performed for 4 biological replicates.

The analysis of glutathione (GSH) and its precursors' content was performed according to (Le Faucheur et al., 2006). Plant samples were homogenized in a bead mill in the presence of 0.1 % trifluoroacetic acid (w/v) and 6.3 mM diethylenetriaminepentaacetic acid and centrifuged. The supernatants were derivatized in darkness for 30 min. at 45 °C using monobromobimane (mBBr). HPLC analyses were performed using Agilent Technologies 1260 system (Santa Clara, CA, USA) equipped with 1260 Infinity Agilent Quaternary pump G1311B, 1260 Infinity Fluorescence Detector G1321B, 1260 Infinity ALS G1329B Automated Sample Injector, 1290 Infinity Autosampler Thermostat G1330B, and a thermostatted column oven 1290 Infinity TCC G1316C. The system was controlled by Agilent OpenLab ChemStation software. GSH and its precursors (cysteine, γ-glutamylcysteine) were separated with a Cosmosil Packed Column C18-MS-II (4.6 µm × 250 mm, 5 µm) and kept at 37 °C with a column oven. Samples (25 µl) were injected and run in a gradient: 0–15 min, 12–25 % (v/v) MeOH; 15–29 min, 25–35 % (v/v) MeOH; 29–50 min, 35–50 % (v/v) MeOH. The column was then cleaned with 100 % (v/v) MeOH and reequilibrated in 12 % (v/v) MeOH. The excitation wavelength was 380 nm; the emission wavelength was 470 nm. The retention times of the analyzed compounds were verified by their standards (AnaSpec, EGT Corporate, USA). To estimate the content of cysteine, γ-glutamylcysteine, and GSH, calibration curves were prepared to quantify the peak areas. Evaluation was performed for 4

biological replicates.

2.13. Determination of carotenoid content

Plant samples were homogenized in 1 ml of methanol in a bead mill and centrifuged. The methanolic extracts were filtered using 0.45 μm , PTFE, HPLC syringe cartridge filters and analyzed in Agilent 1260 Infinity Series HPLC system equipped with Eclipse XDB C8 column (150 mm $\times$ 4.6 mm, 5 μm) at 25 °C. 300 μl of the sample was injected and run in a linear gradient starting from 100 % of eluent A and returning to 100 % at the 40th minute of the analysis. Mobile phase A was a 50/25/25 (v/v/v) mixture of methanol, acetonitrile, and 0.25 M aqueous pyridine solution (pH 5.0) while mobile phase B was a 20/60/20 (v/v/v) mixture of methanol, acetonitrile, and acetone. Absorbance spectra were recorded using a photo-diode array detector (DAD) at 450 nm by Agilent OpenLab ChemStation software (Talarek-Karwel et al., 2020). Evaluation was performed for 3 biological replicates.

2.14. Determination of fatty acids content

Fatty acids from 100 mg of lyophilized plant tissue were extracted according to (Van Gerpen and Knothe, 2010) in 0.5 ml of hexane with 1 % methanol-potassium hydroxide mixture to get fatty acids methyl esters (FAMES) as products of the reaction of transesterification. The process of fatty acids extraction was augmented by the use of an ultrasonic cleaning bath (Sonorex Digital 10P, Germany) for 90 min at 60 °C. 3 ml of hexane was added to the mixture in 3 parts and evaporated in the presence of nitrogen. Then, the extract was redissolved in 1 ml of hexane. Analyses were performed by a gas chromatograph (7890B GC System) with a mass selective detector MSD5977A (Agilent Technologies, USA). 1 μl of each sample was injected via an Agilent 7683 Injector and Sample tray Series with a split ratio of 30:1. The temperature of the injector and transfer line were kept at 260 °C. A Select HP-88 capillary column (100 m $\times$ 0.25 mm, 0.20 μm , 5 in cage, Agilent Technologies, USA) using helium as a carrier gas (1 ml $\bullet$ min⁻¹) was used. The GC temperature program started at 140 °C (hold time 5 min) and was increased to 240 °C at a ramp rate of 4 °C min⁻¹. The electron energy was 70 eV with the ion source temperature set to 250 °C. Through the run, retention times (RT) and fragment ions including m/z 55.1, 67.1, 79.1, 74.1, 81.1, 87.1, and 99.1 for FAMES were logged (Staszak et al., 2023). Evaluation was performed for 4 biological replicates.

2.15. Statistical analyses

The data underwent analysis and visualization using GraphPad Prism 10 software (GraphPad Software, Boston, MA, USA). Normal distribution of the data were verified using the Shapiro-Wilk test. Group distinctions were assessed through One-Way ANOVA, followed by *post hoc* Šidák's multiple comparisons test. Calculations of IC₅₀ concentration for each tested species were performed on normalized to control root length increments using non-linear regression available in GraphPad in a built-in dose-response module. Statistical differences between the experimental series for which the IC₅₀ was chosen were evaluated using Student's *t*-test. Statistical significance, denoted by asterisks (*) or hash symbols (#), was attributed to *p*-values $\leq$ 0.05.

3. Results

3.1. Primary root growth inhibition - determination of IC₅₀ concentration of SEO

Primary root growth depends on cell proliferation activity in meristematic tissue and cell elongation above the meristematic zone. Therefore, to determine the IC₅₀ concentration of SEO, we used a morphometric parameter – total root length increment.

The root length increment of the four tested species was measured

after 24-hour incubation in SEO at concentration ranging from 0.01–0.05 % for *V. faba*, *L. luteus* and *B. napus* and 0.004–0.009 % for *A. thaliana* (Fig. 1, 2). At concentrations used for the other species, *A. thaliana* did not exhibit root elongation (data not shown). Seedlings incubated in water and in dissolved emulsifier at the concentration used for SEO emulsification were considered as two types of controls: Control and emulsifier (E).

Due to the hydrophobic nature of essential oils, their emulsification is commonly recommended (Verdeguer et al., 2020). Application without an emulsifying agent leads to the formation of oil droplets on the water surface, preventing uniform essential oil contact with the incubated roots. As a result, roots located directly beneath or near a large oil droplet exhibit markedly inhibited growth, while those positioned further away – on the same Petri dish – often grow more vigorously, since oil droplets tend to coalesce over time, concentrating in one area. Emulsification thus enhances essential oil stability, bioavailability, and biological properties, while facilitating its sustainable release (Raveau et al., 2020). To prevent uneven contact of seedlings with SEO and ensure result reproducibility, only the emulsified form of SEO was tested, and preliminary analyses involving the non-emulsified form were discontinued.

The chosen concentration ranges were optimal for IC₅₀ determination of SEO using nonlinear regression (Fig. 2A'-D'). Root growth changed in a dose-dependent manner, and among the tested concentrations, we identified those not affecting root growth (0.01 % for *V. faba* and *L. luteus*), those inhibiting growth by about 50 % (0.01 % for *B. napus* and 0.004 % for *A. thaliana*) as well as those significantly or entirely stopping root elongation (0.009 % for *A. thaliana* and 0.04 % for others) comparing to the control (Fig. 1, 2).

Root growth stimulation, was demonstrated for only one species (*L. luteus*; 151 % of control at 0.01 % SEO concentration; Fig. 1B-B'', 2B) and is the subject of separate research. For IC₅₀ calculations, the concentration causing growth stimulation in *L. luteus* was omitted (Fig. 2B').

The calculated IC₅₀ concentrations of SEO for Fabaceae (0.0286 % *V. faba* and 0.0241 % *L. luteus*) and the substantially lower values for Brassicaceae (0.01 % *B. napus* and 0.0048 % *A. thaliana*) indicate higher potency towards weeds during Fabaceae cultivation (Fig. 2A'-D'). These results reveal species-specific sensitivity to SEO and confirm its selective phytotoxic potential depending on plant taxonomy.

Reduced primary root growth under the influence of emulsified SEO at the IC₅₀ concentration (determined separately for each of the four species) corresponds with changes in other growth parameters such as fresh and dry weight. After 24-h incubation, a statistically significant decrease in root fresh and dry weight was noted compared to control plants (incubated in water or dissolved emulsifier; Fig. 3A-D). The reduction in fresh and dry weight of roots (FW/DW) was 33/50 % for *V. faba*, 51/60 % for *L. luteus*, 37/22 % for *B. napus*, and 56/57 % for *A. thaliana*, respectively. Despite the uniform reduction in primary root length across species, the extent of fresh and dry weight reduction varied considerably, indicating species-specific differences in biomass accumulation under SEO exposure.

3.2. Viability of root cells

Primary root growth inhibition under SEO treatment may result from cell membrane damage leading to decreased cell viability, as demonstrated by increased uptake of Evans blue dye. Spectrophotometric measurements of root viability (Fig. 4A-D) revealed increased absorbance and color intensity in all species treated with SEO at the IC₅₀ concentration. Higher absorbance values were recorded for Brassicaceae roots, along with increased dyeability in response to the emulsifier. Therefore, Fabaceae roots exhibited greater viability after 24 hours of IC₅₀ SEO treatment than Brassicaceae ones, confirming a species-specific response to SEO. This pattern further supports the trend of selective phytotoxicity already observed in root growth inhibition assays.

To further verify the extent of membrane damage induced by SEO,

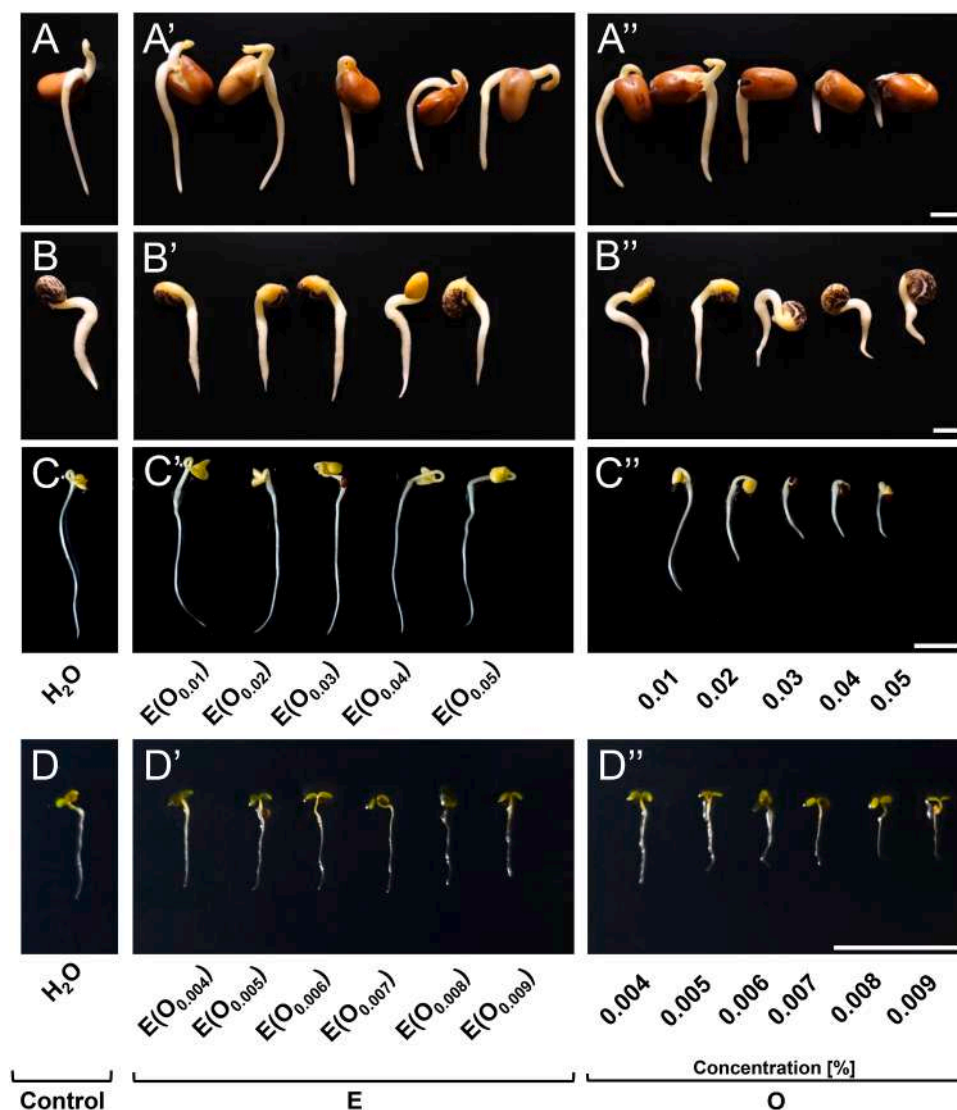


Fig. 1. Seedlings of A-A'') *V. faba*; B-B'') *L. luteus*; C-C'') *B. napus*; D-D'') *A. thaliana* after 24-hour incubation in water – Control, a range of concentrations of emulsifier – E (A'-D'), and essential oil – O (A''-D''). Scale bar: 10 mm.

conductometric measurements were performed. Conductometry is one of the fastest methods for evaluating membrane integrity, as it measures the conductivity of the medium in which seedlings were incubated under sterile conditions (Fig. 5A-D). An increase in conductivity due to ion outflow from damaged cells serves as an indicator of membrane disruption. The analysis revealed elevated conductivity levels in the culture media of all tested species exposed to SEO. However, a significantly lower conductivity was observed in Fabaceae compared to Brassicaceae, indicating that the oil at the IC₅₀ concentration caused less severe membrane damage in the former group. This observation is consistent with the species-specific differences previously described.

3.3. Oxidative stress response

A 24-hour SEO effect is related to increased hydrogen peroxide (H₂O₂) content in primary roots, as proved by spectrophotometric measurements. The highest H₂O₂ content was observed in Fabaceae (Fig. 6A-B) (267 % of control in *V. faba*, 340 % in *L. luteus*) while lower, not exceeding 200 % of control, was observed in Brassicaceae (Fig. 6C-D). Moreover, in *A. thaliana*, a slight but statistically non-significant increase in H₂O₂ level was observed already under emulsifier treatment (Fig. 6D).

Other forms of reactive oxygen species (ROS) can be produced during oxidative stress. One of these is superoxide anion radical (O₂^{•-}) converted by superoxide dismutase (SOD) to oxygen and H₂O₂. SOD activity measurements in emulsifier and SEO-treated seedlings did not exhibit spectacular alterations compared to the control. A slightly increased level of enzyme activity was only observed in emulsifier-treated *L. luteus* roots (Fig. 6B') and under SEO treatment in *V. faba* and *B. napus* (Fig. 6A', C').

Aside SOD and other enzymes maintaining ROS homeostasis in cells, the non-enzymatic antioxidants participate as well. One of them is ascorbate (ASC), taking part in the first stage of a fast and direct neutralization of H₂O₂, acting before enzymes become substrate-saturated and supporting enzymes later on. Control Fabaceae roots exhibit lower ASC levels (of about 1/3 per 1 g FW) than control Brassicaceae roots (Fig. 7A, B and C, D). Ascorbate content measurements revealed two family-specific patterns of seedlings' responses to stress factors like emulsifier and SEO. In the roots of Fabaceae seedlings, the ASC level significantly increased in response to the emulsifier (to around 3 µg·g⁻¹ FW; Fig. 7A, B and C, D). However, in response to SEO, the ascorbate level slightly exceeded the control level (within 2 µg·g⁻¹ FW) and remained significantly lower than that generated by the action of the emulsifier

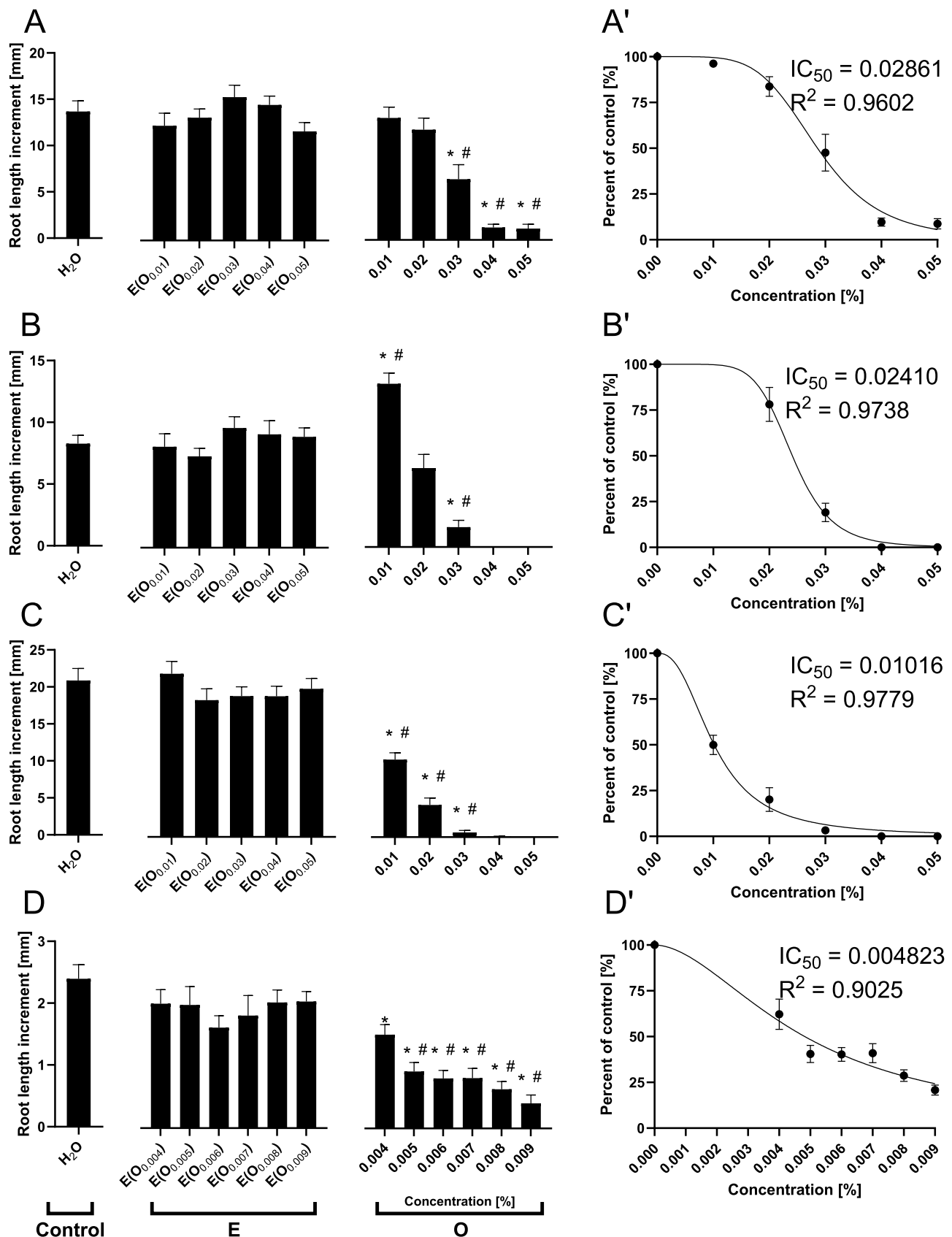


Fig. 2. Increments in root length of A, A') *V. faba*; B, B') *L. luteus*; C, C') *B. napus*; D, D') *A. thaliana* after 24-hour incubation in water – Control, a range of concentrations of emulsifier – E, and essential oil – O. Data are presented as means $\pm$ SEM from three biological replicates ($n = 15$ for A-C and $n = 45$ for D). Statistical differences ($p \leq 0.05$) between experimental series and control were evaluated using one-way ANOVA followed by Dunnett's multiple comparisons test (denoted by asterisks, *). Differences between essential oil and the corresponding emulsifier concentration were assessed by Student's *t*-test and indicated by hash marks (#). Dose-response non-linear curves (A'-D') show root length as percentage of control for seedlings treated with essential oil, based on three biological replicates.

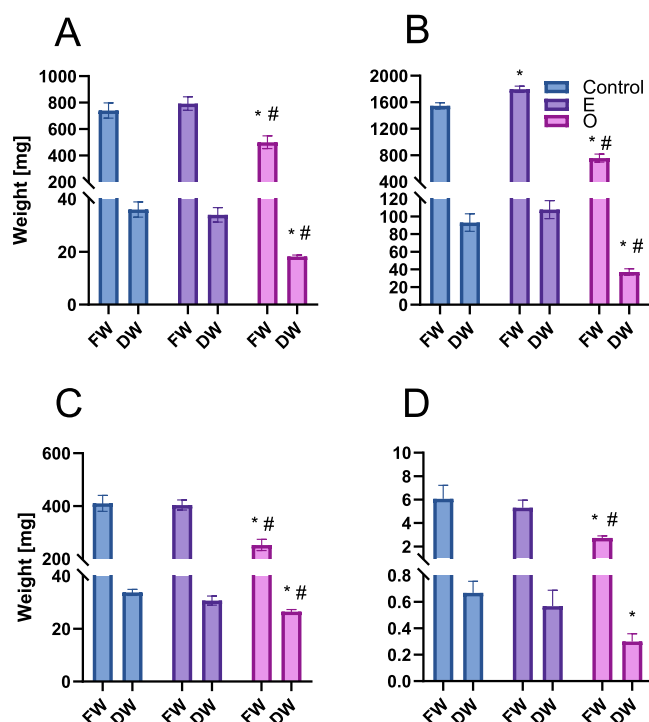


Fig. 3. Fresh and dry weight (FW and DW, respectively) of A) *V. faba*; B) *L. luteus*; C) *B. napus*; D) *A. thaliana* primary roots after 24-hour treatment with emulsifier – E, and essential oil – O at IC₅₀ concentration. Data are presented as means $\pm$ SEM from three biological replicates. Statistical differences were assessed using Student's *t*-test ($p \leq 0.05$). Asterisk (*) and hash marks (#) indicate significant differences compared to the control and the emulsifier treatments, respectively.

(Fig. 7A, B; in these SEO-treated roots, the H₂O₂ level was high Fig. 6A, B). In contrast, in Brassicaceae, the ascorbate resources decreased under emulsifier treatment compared to the control (to about 2.2 $\mu\text{g}\cdot\text{g}^{-1}$ FW Fig. 7C, D; control level of H₂O₂ was maintained only in *B. napus* and remained elevated in *A. thaliana* Fig. 6C, D). However, under SEO, the ascorbate content did not change statistically compared to the control (around 3 $\mu\text{g}\cdot\text{g}^{-1}$ FW) and only moderately increased in *A. thaliana* compared to the emulsifier-treated roots (from 2 to 3 $\mu\text{g}\cdot\text{g}^{-1}$ FW; Fig. 7C, D, despite significant H₂O₂ accumulation, Fig. 6C, D).

As a result of ascorbate level depletion, the ascorbate peroxidase (APX) is locally activated in cells, which uses the remaining ascorbate to more effectively remove H₂O₂, for example, in plastids or mitochondria. In SEO-treated roots of all tested species, increased APX activity was observed (Fig. 7A'-D'), with values rising from 25 to 30 U $\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$ in *V. faba* (Fig. 7A') and from 45 to 55 U $\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$ in *L. luteus* (Fig. 7B'). Moreover, the increased APX activity in Fabaceae was related to decreased ascorbate content (Fig. 7A, B). In Brassicaceae, a statistically significant increase was observed only in *B. napus* (from about 30 to 35 U $\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$; Fig. 7C').

Glutathione (GSH) is the second non-enzymatic antioxidant facilitating the H₂O₂ neutralization and regeneration of other oxidized forms of antioxidants. Its level was similar in control roots of all tested species (within 3–4 $\mu\text{mol}\cdot\text{g}^{-1}$ FW; Fig. 8A-D). A slight increase in glutathione content was only observed in SEO-treated *V. faba* (from 3.3 to just over 4 $\mu\text{mol}\cdot\text{g}^{-1}$ FW; Fig. 8A). However, in both Brassicaceae species, a significant GSH level increase was observed in emulsifier-treated roots (by about 1 $\mu\text{mol}\cdot\text{g}^{-1}$ FW) and retained after SEO treatment (Fig. 8C, D).

The glutathione reductase (GR), which regenerates GSH from glutathione disulfide (GSSG), is responsible for maintaining a proper GSH level in cells and facilitates ROS scavenging. In the control roots of all tested species, the enzyme activity was similar (around 4 U $\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$; Fig. 8A'-D'). After SEO treatment, about a 30–40 %

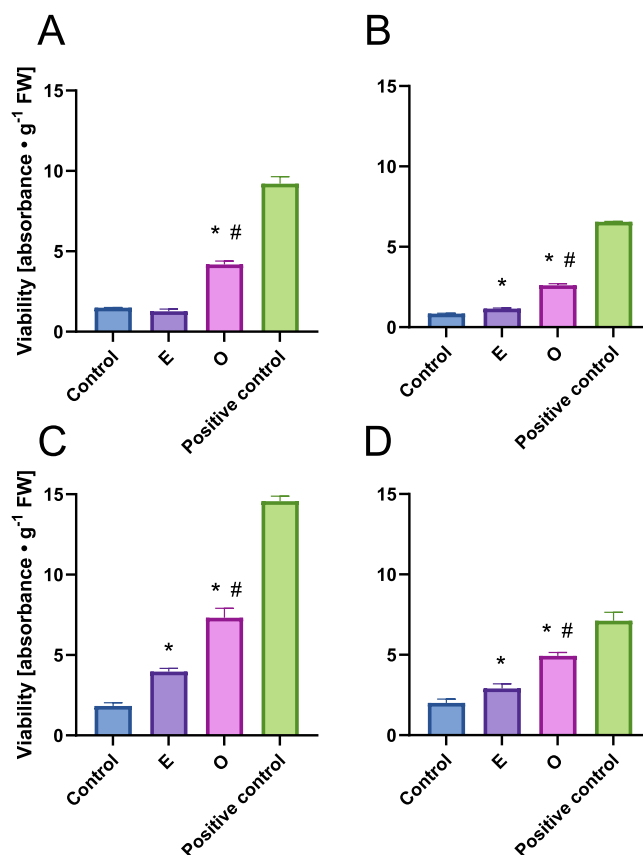


Fig. 4. Loss of cell viability in roots of A) *V. faba*; B) *L. luteus*; C) *B. napus*; D) *A. thaliana* after 24-hour treatment with water – Control, emulsifier – E, and essential oil – O at IC₅₀ concentration. Roots treated with 10 % H₂O₂ for 1 hour were used as a positive control. Data are presented as means $\pm$ SEM from four biological replicates. Statistical differences were assessed using Student's *t*-test ($p \leq 0.05$). Asterisk (*) and hash marks (#) indicate significant differences compared to the control and the emulsifier treatments, respectively.

increase in enzyme activity was observed compared to control or emulsifier-treated seedlings (Fig. 8A'-D').

With an increased demand for GSH, the glutathione two-step biosynthesis pathway from cysteine (Fig. 9A-D) and γ -glutamylcysteine (Fig. 9A'-D') at the appropriate stage is stimulated. In control roots of the tested species, the content of both compounds oscillated on similar levels (± 1 μmol cysteine per g FW and ± 3 μmol γ -glutamylcysteine per g FW). In every species, the emulsifier contributed to a certain increase of cysteine and γ -glutamylcysteine in *V. faba* and *B. napus* (Fig. 9A, C and A', C'). Whereas SEO compared to the emulsifier caused a slight reduction in cysteine content in Fabaceae and a slight increase in Brassicaceae (in both cases by approximately 0.5 $\mu\text{mol}\cdot\text{g}^{-1}$ FW; Fig. 9A, C). The γ -glutamylcysteine content under SEO treatment increased only slightly compared to the control (by approximately 0.5 $\mu\text{mol}\cdot\text{g}^{-1}$ FW; Fig. 9A'-D'), while no clear or consistent response was observed compared to the emulsifier-treated seedlings.

Non-enzymatic ROS scavenging may be facilitated by pigments, among which a significant role is contributed to carotenoids. They quench singlet oxygen and, while being simultaneously oxidized, act as signaling compounds. In roots, their amount are very low (α -carotene around 0.5 $\mu\text{g}\cdot\text{g}^{-1}$ FW; Fig. 10A-D, and β -carotene around 1–2 $\mu\text{g}\cdot\text{g}^{-1}$ FW; Fig. 10A'-D'), but our research has shown alterations in α -carotene and β -carotene content under the influence of the tested factors. The amount of α -carotene significantly decreased already under the influence of the emulsifier alone (by approximately 25 % u *L. luteus* and 75 % in the remaining species; Fig. 10A-D), while the level of β -carotene remained unchanged (Fig. 10A'-D'). SEO treatment in *V. faba*, *B. napus*, and

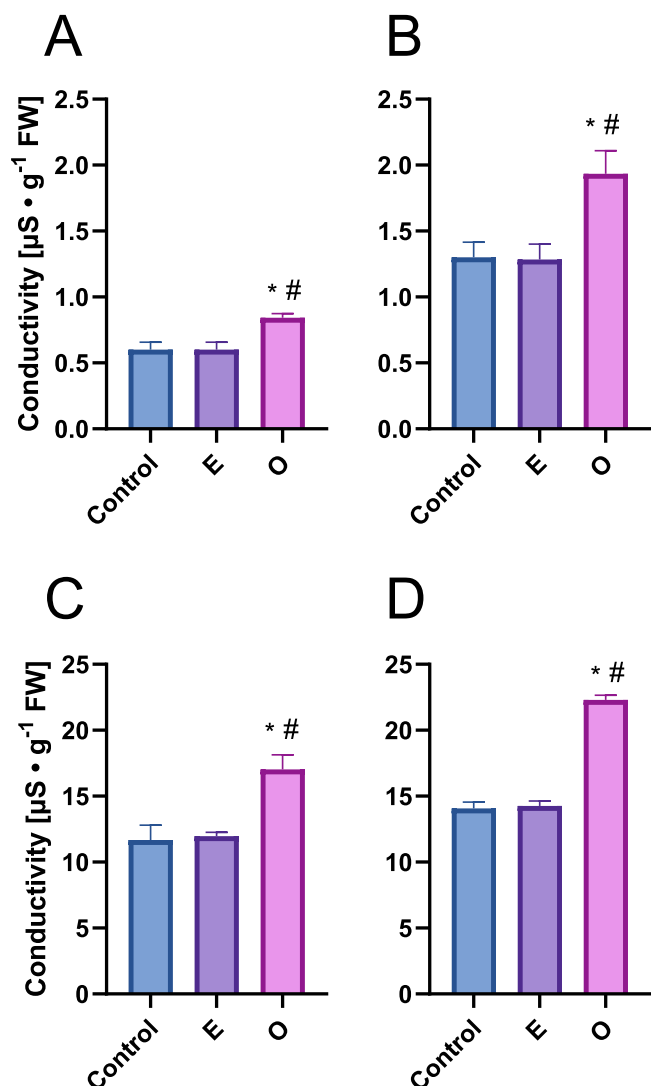


Fig. 5. Conductivity of the growing media of A) *V. faba*; B) *L. luteus*; C) *B. napus*; D) *A. thaliana* after 24-hour treatment with water – Control, emulsifier – E, and essential oil – O at IC₅₀ concentration. Data are presented as means $\pm$ SEM from three biological replicates. Statistical differences were assessed using Student's *t*-test ($p < 0.05$). Asterisk (*) and hash marks (#) indicate significant differences compared to the control and emulsifier treatments, respectively.

A. thaliana reduced the content of the α -carotene almost to zero (Fig. 10A, C, D) and that of β -carotene to about 60–70 % of the control level (Fig. 10A', C' D'). Root cells of *L. luteus* responded differently to SEO, with carotenoid levels noticeably increasing compared to the emulsifier effect (by about 50 % α -carotene and 30 % for β -carotene; Fig. 10B, B').

At sustained high H₂O₂ concentrations, catalase (CAT) activity also contributes to cells against increased levels of this harmful ROS. In control plants, CAT activity was around 30 U·mg protein⁻¹·min⁻¹ (Fig. 11A–D). Treatment with the emulsifier caused a slight decrease in CAT activity compared to the control, except in *L. luteus*, where a slight increase was observed (compare Fig. 11A, C, D and B). After SEO treatment, a slight increase in CAT activity was noted in the roots of all tested species (by approximately 3–5 U·mg protein⁻¹·min⁻¹; Fig. 11A, B, D), with the highest increase observed in *B. napus* (about 8 U·mg protein⁻¹·min⁻¹; Fig. 11C).

The observed biochemical responses indicate that Fabaceae roots react to SEO-induced oxidative stress with a more dynamic and efficient antioxidant defense system compared to Brassicaceae, despite exhibiting

higher H₂O₂ levels. These observations further highlight species-specific physiological responses to SEO treatment, indicating its selective phytotoxicity and supporting its potential use in targeted weed control strategies.

3.4. Metabolic activity

Isothermal calorimetry is a useful tool to monitor metabolic heat emission during germination or seedling growth and has found use in allelopathy research. The analysis based on heat emission or absorption measurements in plant samples gives a real-time response of plant metabolism to the tested factor (Fig. 12). The seedlings incubated in an emulsifier solution in the concentration used to emulsify the SEO in IC₅₀ were a control. During the experiment, seedlings treated with the emulsifier quite stably emitted heat on a species-specific level (Fig. 12A, B, D), except for *B. napus* seedlings, in which heat emission gradually increased (Fig. 12C). The highest heat emission was recorded in Brassicaceae (approximately 1–2 mW per g FW for *B. napus* and about 3 mW per g FW for *A. thaliana*; Fig. 12C, D) and about 6 times lower for Fabaceae seedlings (0.5 mW per g FW for *V. faba* and 0.3 mW per g FW for *L. luteus*; Fig. 12A, B). The progress of thermal curves changed during the seedlings' exposition to SEO. *V. faba* seedlings responded with sudden, robust, and 2 times higher heat emission than emulsifier-treated seedlings, stable to the end of the analysis (Fig. 12A). Heat emission by *L. luteus* seedlings gradually increased from the low-level peculiar to emulsifier-treated seedlings up to 1 mW per g FW at the end of the experiment (Fig. 12B). Heat emission by *B. napus* seedlings followed a similar growth pattern to that observed under the influence of the emulsifier but occurred at a significantly higher level (Fig. 12C). Surprisingly, *A. thaliana* seedlings respond to SEO differently. Throughout the analysis, they absorbed heat on a stable level (oscillating near –20 mW per g FW) while a strong endothermic peak was recorded at the 20th hour of incubation in SEO (Fig. 12D). These results point to different metabolic strategies of Fabaceae and Brassicaceae in response to SEO. Fabaceae display more controlled and sustained metabolic responses, whereas Brassicaceae undergo pronounced shifts that may indicate less efficient metabolic adaptation under stress. Perturbations in plant metabolism can be related to the altered content of essential components such as proteins, sugars, or fatty acids.

Analysis of protein content using the Bradford method did not reveal any alterations in emulsifier-treated roots across all tested species (Fig. 13A–D). In contrast, SEO treatment generally led to a decrease in protein levels in Fabaceae roots and an increase in Brassicaceae (compare Fig. 13A, B and C, D). However, a distinct and statistically significant decrease was observed in *L. luteus* (about 20 %; Fig. 13B), while a moderate but statistically significant increase was noted in *B. napus* (about 15 %; Fig. 13C).

On the other hand, monosaccharide content analysis revealed distinct changes in Fabaceae roots under emulsifier treatment, with values nearly doubling compared to the control in both species (Fig. 13A', B'). This suggests a strong stimulation of sugar accumulation as a potential short-term metabolic adaptation. In contrast, Brassicaceae roots exhibited a slight decrease in monosaccharide content under emulsifier exposure, although the change was statistically significant only in *B. napus* (Fig. 13C', D'). SEO treatment did not result in a clear family-specific trend. Compared to the emulsifier series, a distinct reduction in monosaccharide levels was observed in *V. faba* (about 35 %; Fig. 13A'), while a slight reduction was noted in *B. napus*, (about 15 %; Fig. 13C'). In contrast, *L. luteus* and *A. thaliana* roots responded with a moderate increase (about 25 %) and a marked increase (about 35 %), respectively (Fig. 13B', C'). These results suggest a species-specific adjustment of carbohydrate metabolism in response to SEO exposure, potentially reflecting differences in energy demand or metabolic prioritization under stress.

Analysis of individual fatty acid groups showed that both the emulsifier and the SEO significantly affected their content (Fig. 14A–D). Fatty

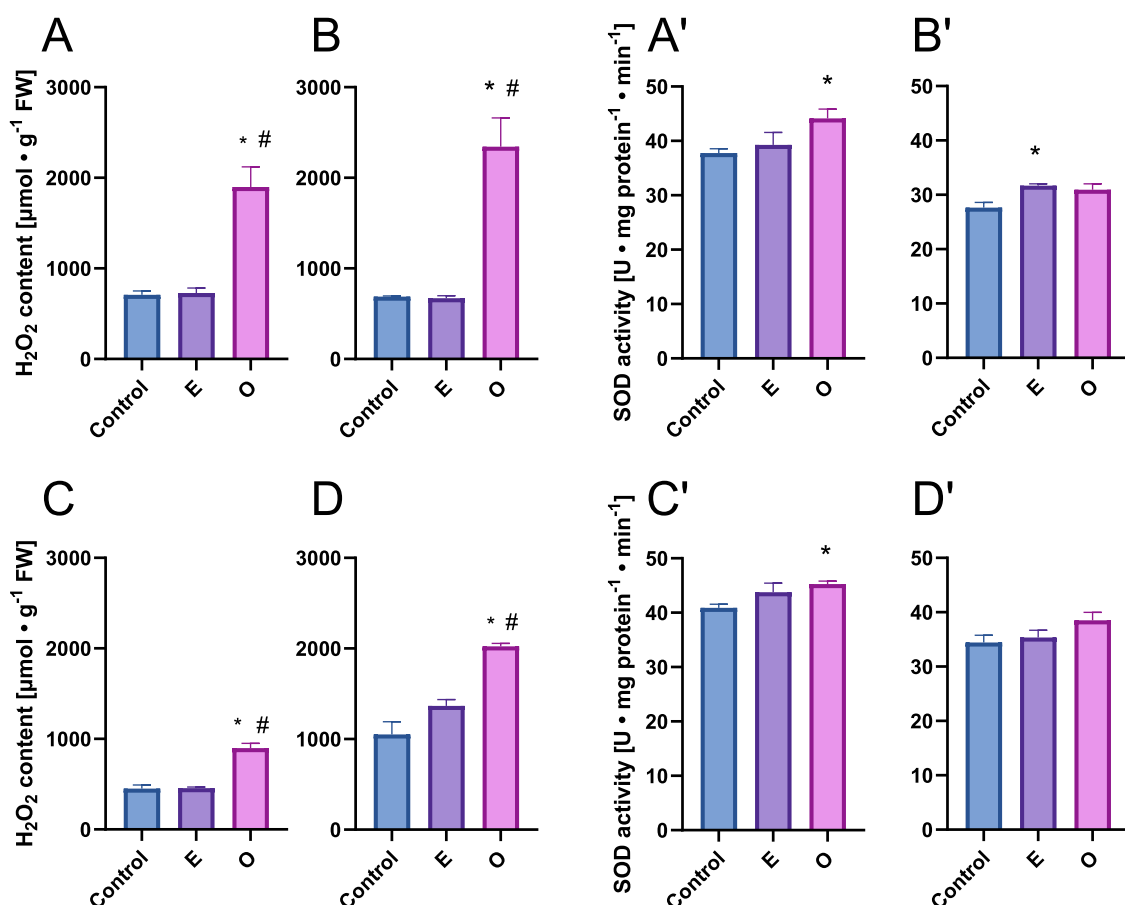


Fig. 6. Hydrogen peroxide (H₂O₂) content (A–D) and superoxide dismutase (SOD) activity (A'–D') in primary roots of A, A') *V. faba*; B, B') *L. luteus*; C, C') *B. napus*; D, D') *A. thaliana* after 24-hour treatment with water – Control, emulsifier – E, and essential oil – O at IC₅₀ concentration. Data are presented as means ± SEM from four biological replicates. Statistical differences were assessed using Student's *t*-test (*p* ≤ 0.05). Asterisk (*) and hash marks (#) indicate significant differences compared to the control and the emulsifier treatments, respectively.

acids were grouped into saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) forms. However, no consistent pattern of response was observed across families. Emulsifier treatment influenced the content of individual fatty acid groups in a species-specific manner. In general, a decrease in SFA levels was observed, particularly pronounced in *Fabaceae* (Fig. 14A, B). In contrast, *Brassicaceae* exhibited an increase in SFA content (Fig. 14C, D). A clear rise in MUFA levels was recorded in *V. faba* as well as in the *Brassicaceae* species, whereas in *L. luteus*, MUFA content remained relatively unchanged. PUFA levels increased in *V. faba* and *A. thaliana*, while a decrease was noted in *L. luteus* and *B. napus*. SEO treatment in *V. faba* (Fig. 14A), led to a nearly threefold increase in SFA content (up to approximately 10 mg·g⁻¹ DW), accompanied by a marked reduction in MUFA and PUFA levels (approximately 50 %; to about 6 and 7.5 mg·g⁻¹ DW, respectively) compared to the emulsifier-treated roots. In contrast, *L. luteus* (Fig. 14B) responded oppositely, with a substantial (~50 %) decrease in SFA (to around 3 mg·g⁻¹ DW) and a concomitant increase in MUFA and PUFA content (approximately 11 and 7.5 mg·g⁻¹ DW, respectively). Among *Brassicaceae*, *B. napus* (Fig. 14C) displayed the most prominent changes: a several-fold increase in SFA content (to about 40 mg·g⁻¹ DW), no change in MUFA (around 20 mg·g⁻¹ DW), and a drastic drop in PUFA levels (down to nearly 1 mg·g⁻¹ DW) under SEO treatment. Meanwhile, in *A. thaliana* (Fig. 14D), SEO treatment caused an overall increase in the content of all fatty acid groups, with SFA rising to approximately 50 mg·g⁻¹ DW, MUFA to 25 mg·g⁻¹ DW, and PUFA to about 45 mg·g⁻¹ DW, compared to the control. Overall, the most pronounced changes in fatty acid profiles were observed in *Brassicaceae* roots, particularly under SEO treatment, although the direction and magnitude of the response

varied between species.

3.5. Lipid peroxidation

A marker of oxidative stress and oxidative damage is malonyl dialdehyde (MDA), one of the final byproducts of lipid peroxidation forming as a result of ROS activities on polyunsaturated fatty acids (PUFA) in cell membranes. The SEO used in the present study increased H₂O₂ production in all tested species and contributed to MDA content increment (compare Fig. 6A–D and Fig. 15A–D). In *Fabaceae*, this increase was relatively moderate, reaching approximately 144 % of the control level in *V. faba* and 122 % in *L. luteus* (Fig. 15A, B). In contrast, in *Brassicaceae*, the increase was significantly higher, with MDA levels reaching about 157 % of the control in *B. napus* and as much as 452 % in *A. thaliana* (Fig. 15C, D). Thus, the extent of lipid peroxidation induced by SEO was considerably lower in *Fabaceae* compared to *Brassicaceae*. This supports the previously observed pattern of more effective oxidative stress mitigation in *Fabaceae*, which experience lower lipid peroxidation despite higher H₂O₂ levels.

4. Discussion

4.1. SEO's selective phytotoxicity

At the initial stage of searching for new compounds with herbicidal potential, it is essential to determine their selectivity, for example, towards monocotyledonous and dicotyledonous plants or specific weed and crop species (Gruřová et al., 2020). Equally important is

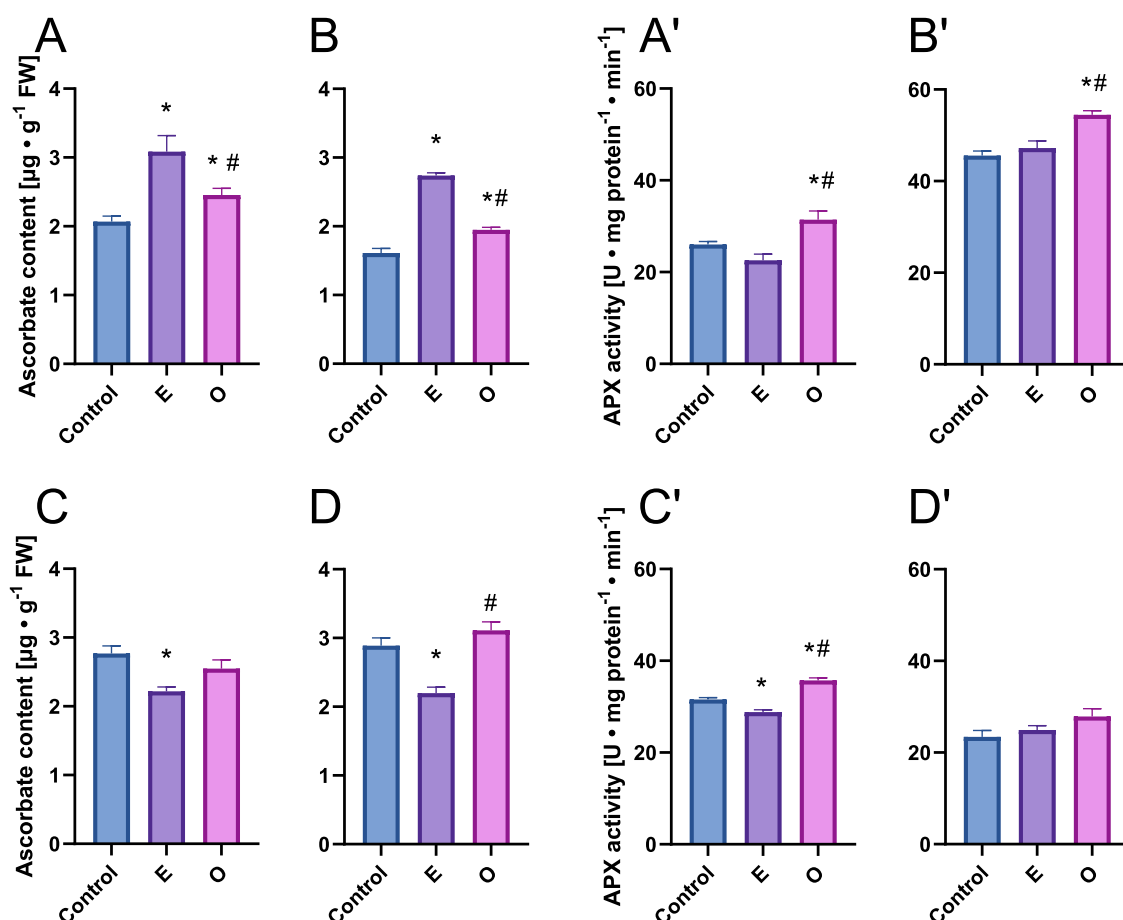


Fig. 7. Ascorbate content (A–D) and ascorbate peroxidase (APX) activity (A'–D') in primary roots of A, A') *V. faba*; B, B') *L. luteus*; C, C') *B. napus*; D, D') *A. thaliana* after 24-hour treatment with water – Control, emulsifier – E, and essential oil – O at IC_{50} concentration. Data are presented as means $\pm$ SEM from three biological replicates. Statistical differences were assessed using Student's *t*-test ($p \leq 0.05$). Asterisk (*) and hash marks (#) indicate significant differences compared to the control and the emulsifier treatments, respectively.

investigating their mode of action, including identifying the disruptions they cause in plant functioning and determining the metabolic pathways they affect. Such an analysis forms the foundation for evaluating the efficacy and safety of new substances in crop protection. In the present study, four plant species were selected for analysis: two belonging to the Fabaceae family, which includes many crop species, and two from the Brassicaceae family, often represented by species considered as weeds. In the first phase of the research, the IC_{50} concentrations were determined for each tested species (0.03 % for *V. faba*, 0.025 % for *L. luteus*, 0.01 % for *B. napus*, and 0.05 % for *A. thaliana*). The obtained results indicated the ability to delineate SEO concentration ranges that significantly affect Brassicaceae growth and do not impact Fabaceae seedlings or, moreover, can stimulate the growth observed in *L. luteus* seedlings treated with 0.01 % SEO (the observed phenomenon of growth stimulation is a topic for other studies).

Comparing differences in SEO concentration ranges, which significantly affect root length increment, they enormously differ between Fabaceae and *A. thaliana* and much less for Fabaceae and *B. napus*. However, the obtained results allow us to assume that the application of determined SEO for other cultivated Fabaceae in order to eliminate Brassicaceae as well as other weed species can give results that are selective in nature. Optimistic confirmation is brought by studies evaluating the impact of SEO on *Lactuca sativa* L. and *Lolium perenne* L. germination and seedling growth (Satyal et al., 2013). The authors determined significantly different IC_{50} values characteristic for germination inhibition of both species ($450 \mu\text{g ml}^{-1} = 0.045$ % for *L. sativa* and $737 \mu\text{g ml}^{-1} = 0.0737$ % for *L. perenne*), indicating different

susceptibility. They demonstrated as well reversed species-specific susceptibility of seedlings to applied SEO in a concentration range of $1000\text{--}250 \mu\text{g ml}^{-1}$ (equivalent to 0.1–0.025 %), which mildly inhibited *L. sativa* and strongly *L. perenne* seedling growth. Preliminary selectivity at this seedling developmental stage is important from a practical perspective, as it indicates the possibility of using SEO in a targeted manner – eliminating certain species without affecting others, potentially cultivated ones (Da Costa et al., 2020). Among studies evaluating the root growth of the most abundant weeds such as *Echinochloa crus-galli* L. P. Beauv., *Amaranthus retroflexus* L., *Poa annua* L., and *Avena fatua* L. after direct contact with essential oils, obtained in this study the IC_{50} values (within the range of about (0.03–0.005 %)) place the SEO as one exhibiting the most effective herbicidal activity. In other studies, a 50 % reduction in weed growth occurred after application of 0.2 % *Ambrosia artemisiifolia* L. (Han et al., 2021), 0.05 % *Tagetes erecta* L. (Laosinwattana et al., 2018), 0.05 % *Citrus aurantiifolia* (Fagodia et al., 2017), 0.2–0.1 % *Thymus proximus* Serg. (Zhou et al., 2021), and 0.2 % *Artemisia fragrans* Willd. (Pouresmaeil et al., 2020) essential oils.

At species-specific IC_{50} concentrations – each reducing primary root growth by 50 % – SEO induced distinct reductions in FW and DW (expressed as % of the control: FW/DW was 33/50 % for *V. faba*, 51/60 % for *L. luteus*, 37/22 % for *B. napus*, and 56/57 % for *A. thaliana*), reflecting interspecies differences in physiological responses and tolerance mechanisms under an equivalent level of stress. The results suggest that a relatively greater reduction in FW, as observed in *B. napus*, may indicate disturbances in water balance, such as impaired uptake or dehydration, whereas a more pronounced DW reduction, as seen in

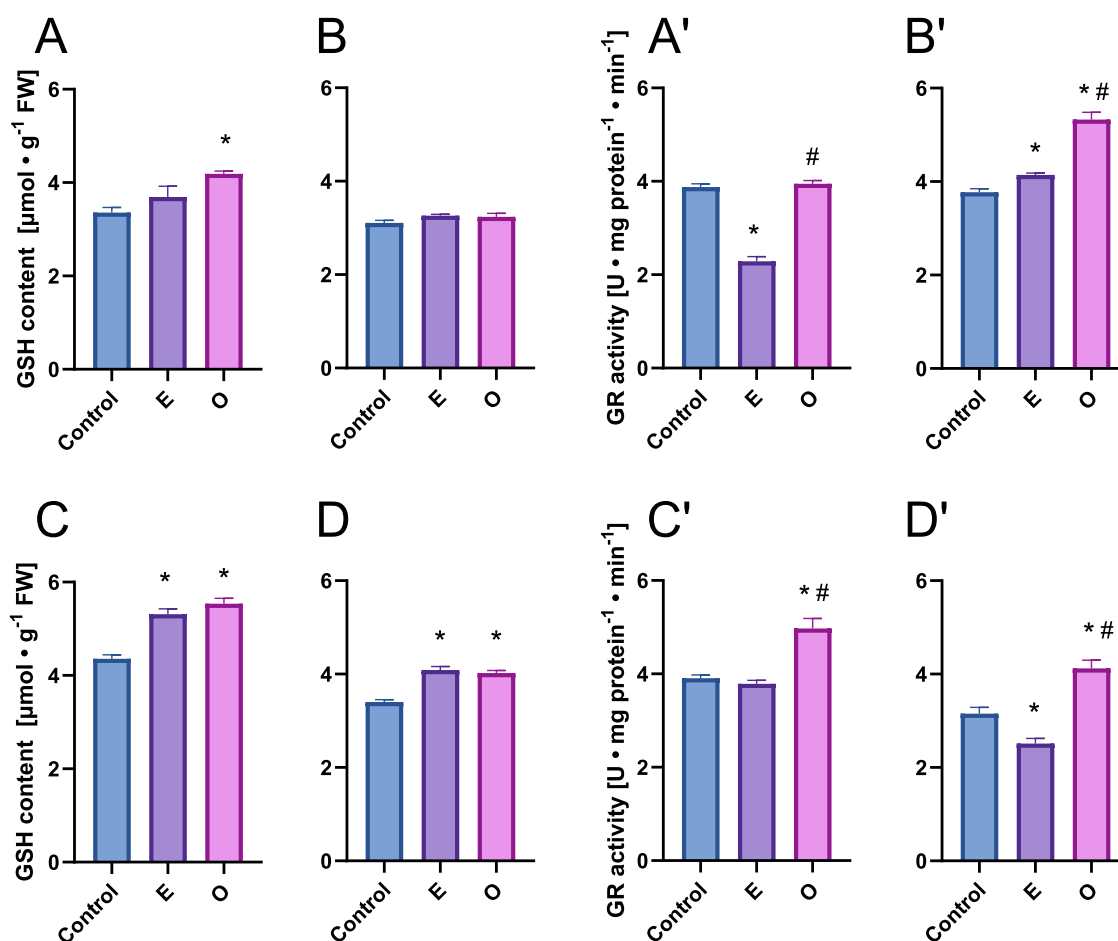


Fig. 8. Glutathione content (A-D) and glutathione reductase (GR) activity (A'-D') in primary roots of A, A') *V. faba*; B, B') *L. luteus*; C, C') *B. napus*; D, D') *A. thaliana* after 24-hour treatment with water – Control, emulsifier – E, and essential oil – O at IC_{50} concentration. Data are presented as means $\pm$ SEM from three biological replicates. Statistical differences were assessed using Student's *t*-test ($p \leq 0.05$). Asterisk (*) and hash (#) indicate significant differences compared to the control and the emulsifier treatments, respectively.

V. faba, could result from suppressed biomass synthesis or metabolic dysfunction, despite relatively maintained hydration. When considering both parameters, *V. faba* appears to respond more moderately than *L. luteus*, which seems even more sensitive than the Brassicaceae representative. In turn, *B. napus* demonstrates greater tolerance than *A. thaliana*. These results confirm the selective action of SEO, although the observed patterns appear to be more species-specific than family-dependent. Our findings correspond with observed fresh and dry weight reductions for several weed species treated with essential oils (Li et al., 2023; Pouresmaeil et al., 2022, 2020).

The inhibition of root growth and the reduction in both fresh and dry biomass observed after the application of different essential oils, including SEO, represent some of the most common and universal plant responses to various stress factors (Coşkun, 2023). These responses occur in reaction to both abiotic stressors – such as drought, salinity, extreme temperatures, nutrient deficiencies, and heavy metal contamination – and biotic stressors, including allelopathy, interspecies competition, and pathogen infection (Aydin and Coskun, 2013a, 2013b; Sahu et al., 2022). Despite their differing nature, many of these stress factors initiate convergent signaling cascades and activate overlapping biochemical pathways (Yoo et al., 2024). This suggests that plant responses to allelopathic compounds, such as components of essential oils, may proceed through universal mechanisms characteristic of environmental stress responses (Aydin and Coskun, 2013a; 2013b). A key component of this response is oxidative stress, which arises secondarily due to the overproduction of reactive oxygen species (ROS). Although plants possess complex antioxidant defense systems – comprising both

enzymatic and non-enzymatic components – these defenses may become overwhelmed under severe or prolonged stress conditions. Consequently, cellular homeostasis is disrupted, leading to impaired organelle function, metabolic disturbances, and structural damage, including membrane lipid peroxidation (Li et al., 2023).

4.2. Oxidative stress response

In light of the observed selective inhibition of root growth depending on SEO concentration, this study aimed to determine whether application of an equivalent stress intensity (i.e., IC_{50} dose adjusted for each species) would trigger similar or divergent (species- or family-specific) oxidative stress responses in the tested plants. The study also investigated whether the four species exhibit comparable or distinct antioxidant system efficiency, whether they activate analogous or different defense components, and whether they undergo similar metabolic alterations, including the extent of lipid peroxidation. The tested in our study SEO concentrations were within the range of regarded as safe for animal cells (Lam et al., 2017).

Each cell compartment contains its pool of non-enzymatic, low molecular weight antioxidants and antioxidative enzymes, forming a highly complex, multi-level defense system warranting ROS homeostasis (Dusart et al., 2019). Redox transitions are dynamic, and the reaction network is extensive and full of dependencies, making the whole response process hard for simultaneous recognition and unbiased interpretation. SEO used in the present study was applied to the growing medium in emulsified form. Though not exhibiting a significant impact

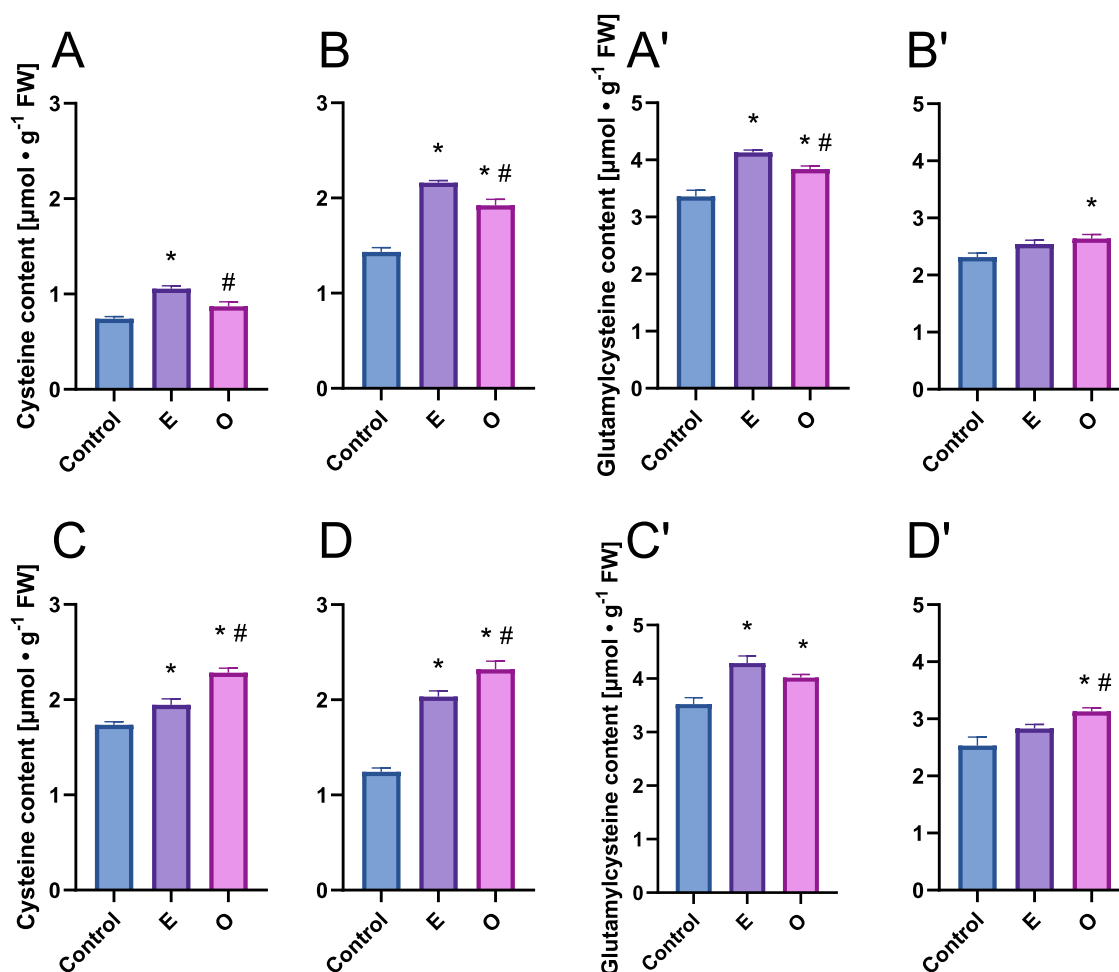


Fig. 9. Cysteine (A–D) and γ -glutamylcysteine (A'–D') content in primary roots of A, A') *V. faba*; B, B') *L. luteus*; C, C') *B. napus*; D, D') *A. thaliana* after 24-hour treatment with water – Control, emulsifier – E, and essential oil – O at IC₅₀ concentration. Data are presented as means $\pm$ SEM from four biological replicates. Statistical differences were assessed using Student's *t*-test ($p \leq 0.05$). Asterisk (*) and hash (#) indicate significant differences compared to the control and the emulsifier treatments, respectively.

on root length increment (compared to control) of all tested species, the emulsifier interfered in cellular redox status as well. In its presence, we noted an increase of non-enzymatic ROS scavengers such as ascorbate in Fabaceae and glutathione (GSH) in Brassicaceae, while in *L. luteus*, the activity of enzymatic antioxidants such as SOD, CAT, and GR was additionally increased. Eventually, under emulsifier treatment, the increased H₂O₂ content featured *A. thaliana* root cells and a slight increase in lipid peroxidation occurred in *V. faba*. Obtained outcomes reveal activation less or more extended (e.g., by SOD, CAT, and GR enzymes) network of antioxidant pathways which, during emulsifier response, efficiently secure cells providing correct (similar to control) seedling growth of the particular species.

Emulsified SEO (compared to emulsifier) used in the concentrations limiting root growth increment by 50 % made spectacular changes in redox status. Consequently, a significant amount of H₂O₂ (although with a minor SOD contribution) accumulated in cells. The amounts in Fabaceae were definitely higher (± 350 % of control); in Brassicaceae, they were relatively lower (not exceeding 200 % of control). As a result of developing oxidative stress in all tested species, the content of non-enzymatic antioxidants or their precursors acting in the ascorbate-glutathione cycle as the first line of defense has changed. Ascorbate functions as an antioxidant by direct chemical interaction with free oxyradicals and in APX-catalyzed reactions. Its oxidation affects the redox balance of other metabolites (such as glutathione), playing a role in cellular redox imbalances detection as well (De Kok et al., 1986;

Kampfenkel et al., 1995; Piotrowska-Niczyporuk et al., 2012; Singh et al., 2006). Admittedly, in Fabaceae, the level of ascorbate decreased (adversely to Brassicaceae), but the activity of APX, which is the ascorbate-utilizing enzyme, increased. In turn, the GSH level in all tested species was maintained in a range peculiar for control or emulsifier-treated seedlings. However, its active participation in anti-oxidative defense is evidenced by improved activity of glutathione reductase (GR) which regenerated GSH from GSSG. Moreover, glutathione is biosynthesized in enzyme-catalyzed reactions from glutamine and cysteine, forming γ -glutamylcysteine, which, in the next step, binds with glycine and forms glutathione (Ito and Ohkama-Ohtsu, 2023). In all tested species, the content of cysteine and γ -glutamylcysteine was relatively high, though slightly lower in Fabaceae compared to emulsifier-treated seedlings. Although the current study did not investigate the ratio of reduced/oxidized ASC and GSH, analysis of ASC and GSH levels, as well as APX and GR activities, confirms the functioning of the ascorbate-glutathione cycle in H₂O₂ scavenging under SEO-induced stress in roots. Supporting compounds in antioxidant protection and in maintaining cellular structure integrity, such as cellular and organellar membranes, are carotenoids. In roots, they are less present than in photosynthesizing tissues and cooperate with low molecular weight antioxidants and enzymes in ROS scavenging (Dumanović et al., 2021). In our studies, the carotenoid content (α - and β -carotene) increased only in *L. luteus*, and in other species, the relatively low carotenoid level decreased under SEO treatment. It is likely that in Fabaceae,

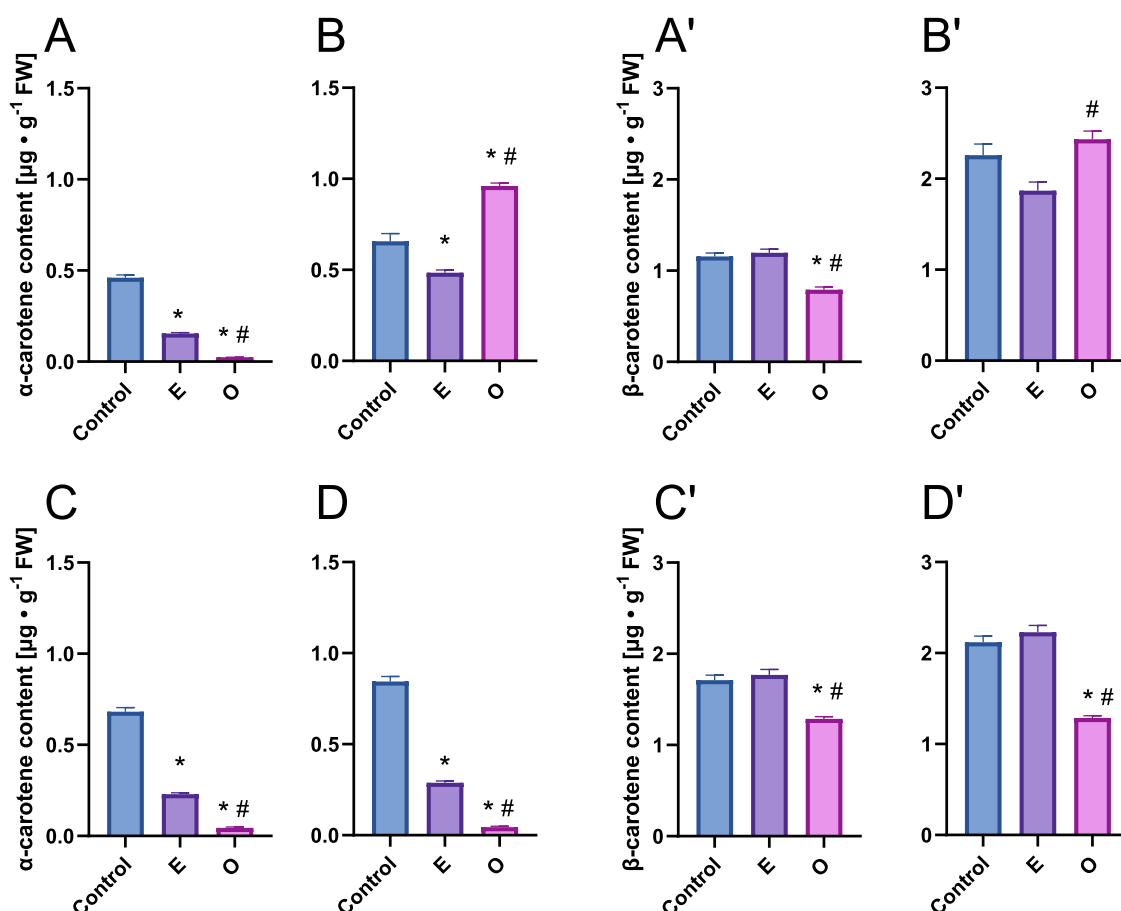


Fig. 10. Content of α - (A–D) and β -carotene (A'–D') in primary roots of A, A') *V. faba*; B, B') *L. luteus*; C, C') *B. napus*; D, D') *A. thaliana* after 24-hour treatment with water – Control, emulsifier – E, and essential oil – O at IC_{50} concentration. Data are presented as means $\pm$ SEM from three biological replicates. Statistical differences were assessed using Student's *t*-test ($p \leq 0.05$). Asterisk (*) and hash (#) indicate significant differences compared to the control and the emulsifier treatments, respectively.

polyphenols and flavonoids (Desoky et al., 2021) whose levels were not analyzed in the present study, may have also contributed to ROS scavenging. When, despite the dynamically functioning non-enzymatic system, the concentration of H_2O_2 still increases to a level necessary to saturate the enzyme, CAT activity is triggered, which more efficiently facilitates the detoxification process (Dumanović et al., 2021). An increase in the enzyme activity was observed in *Triticum aestivum* L. under β -pinene (Chowhan et al., 2014) or *Artemisia scoparia* Waldst & Kit. essential oil exposure (Kaur et al., 2012). Adversely, in plants in which the essential oil concentration inhibited growth by about 50 %, a decrease in CAT, SOD, and APX activity was observed due to the inability of these enzymes to scavenge excessive H_2O_2 having a detrimental effect on cellular components (Pouresmaeil et al., 2022, 2020). In the current study, CAT activity increased in the tested species; however, the enzyme failed with toxic H_2O_2 scavenging. Antioxidant systems, thus effective, are unable to scavenge all the ROS species during stress. Although the involvement and activation of all analyzed oxidative stress and antioxidant defense parameters were demonstrated in the cells of the four tested species, species-specific differences in the magnitude of these responses were also revealed. Such diversity in redox responses suggests that species-specific tolerance to SEO does not solely result from the general activity of the bioactive compounds but rather stems from a complex interaction between oil components and the specific plant's protective system. Literature highlights the species-dependent activity of antioxidant pathways and varying sensitivity to reactive oxygen species (ROS) generated under stress (Araniti et al., 2018a; Chowhan et al., 2014; Khare et al., 2019). Undoubtedly, the differential plant response is an advantage when considering SEO as

a potential bioherbicide candidate aimed at selectively eliminating Brassicaceae plants from Fabaceae crop systems in the future. In this context, it is also important to consider the potential effects of ROS overproduction on genome stability. Excessive ROS production, triggered by compounds envisioned as future bioherbicides, may destabilize the genome by inducing DNA strand breaks (Szurman-Zubrzycka et al., 2023). These lesions can impair meristematic cell proliferation, thus restricting seedling growth (as described for *P. gaudichaudianum* oil; Mendes et al., 2023), or under extreme conditions, activate programmed cell death pathways. Observing greater sensitivity and stress response deficiencies at the levels of genome protection (via epigenetic regulation or DNA repair) in Brassicaceae species compared to Fabaceae would further strengthen the case for SEO as a selective and effective bioherbicide. Preliminary studies in this direction have already been undertaken.

4.3. Metabolic and membrane responses

The loss of redox homeostasis and sustained oxidative stress faced by cells are reflected in their metabolism. Heat emission occurring during plant growth is related to energy transformation, which is emitted as metabolic heat regarded as a byproduct of metabolic processes and can be monitored using isothermal calorimetry (Rys et al., 2024; Skoczowski and Troć, 2013). Moreover, the increase in heat emission may be related to the activation of repair mechanisms and the biosynthesis of protective compounds involved in the detoxification of H_2O_2 (Skoczowski and Troć, 2013). Defense strategies for coping with oxidative stress and affected dynamics of metabolic processes by SEO may interfere with

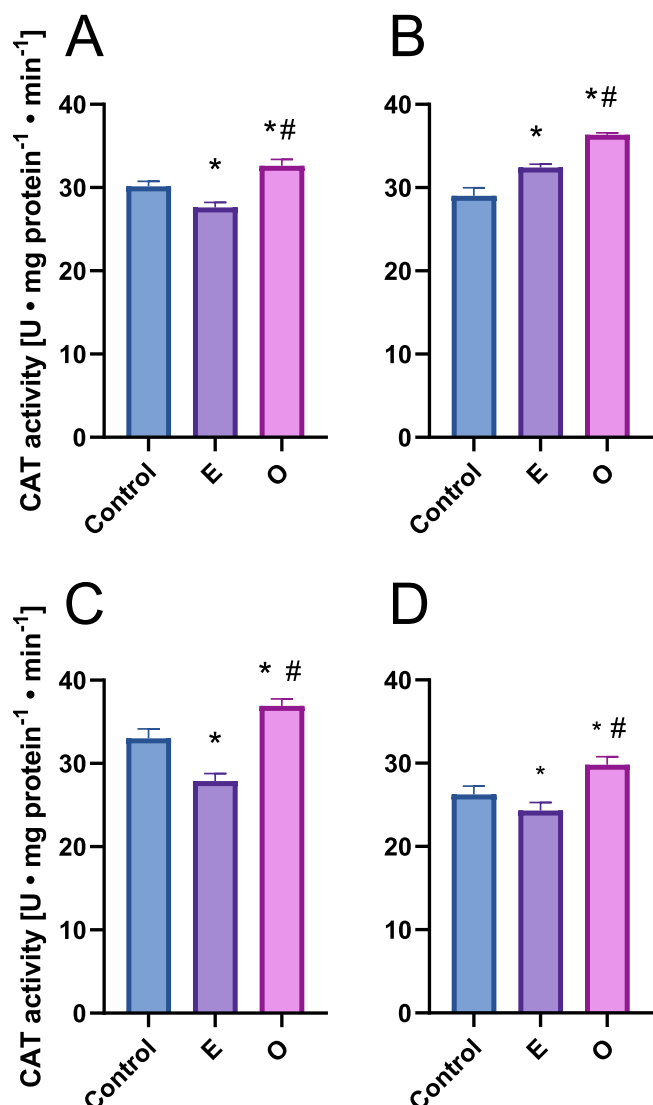


Fig. 11. Catalase activity (CAT) in primary roots of A) *V. faba*; B) *L. luteus*; C) *B. napus*; D) *A. thaliana* after 24-hour treatment with water – Control, emulsifier – E, and essential oil – O at IC_{50} concentration. Data are presented as means $\pm$ SEM from three biological replicates. Statistical differences were assessed using Student's *t*-test ($p \leq 0.05$). Asterisk (*) and hash (#) indicate significant differences compared to the control and the emulsifier treatments, respectively.

heat emission by seedlings or cause heat absorption from the environment. Heat emission is the parameter allowing to evaluate relatively fast-occurring differences or similarities in SEO response between plants classified into different species or families. Our results highlight contrasting metabolic responses to SEO between Fabaceae and Brassicaceae. Fabaceae species exhibit more moderate and controlled changes in specific thermal power, with *V. faba* showing remarkable metabolic stability and *L. luteus* displaying a gradual increase suggestive of an adaptive response. In contrast, Brassicaceae species reveal pronounced and energetically costly shifts – from strong metabolic activation in *B. napus* to deep metabolic suppression in *A. thaliana* – indicating a lower capacity for maintaining metabolic homeostasis under stress. Observed in the first three species, individual trajectories of the heat emission curves may indicate the different plant sensitivity to SEO and the involvement of different mechanisms and stress response pathways, which eventually have led to the common result which is high heat emission. This increase may be related to intense oxidative metabolism in seedlings occurring during SEO treatment, which could be linked with the intensification of catabolic processes such as cellular respiration,

increased mobilization of sugar reserves for the rapid production of monosaccharides and their use in glycolysis and Krebs cycle or with ROS production increase (Saja et al., 2014; Skoczowski and Troć, 2013). An opposite effect, the absorption of heat instead of emission, indicates rather inhibiting metabolic activity. It may have occurred, for instance, due to protein damage involved in these processes. In our studies, *A. thaliana* was the only species in which heat absorption was recorded. A similar phenomenon was observed in *Zea mays* L. and *Echinochloa crus-galli* seedlings incubated in 5 % *Carum carvi* bio-nanoemulsion, which inhibited their growth (Rys et al., 2024). The reduction in thermal power was also observed in *Sinapis alba* L., *Brassica napus* L., and *Trifolium repens* L. seedlings incubated in herbal extracts, and the phenomenon was explained by the loss of membrane integrity during treatment and subsequently, the interaction of root exudates with the herbal extract in the incubation medium (Troć et al., 2011).

Both SEO components and accumulating ROS can affect the protein content in cells by disturbing protein biosynthesis processes (Ootani et al., 2017; Synowiec et al., 2019) or directly damaging already functioning ones (Werrie et al., 2020). At the same time, stress-induced accumulation of protective proteins may lead to an overall increase in cellular protein content (Kosová et al., 2018). Such changes in the complete proteomic profile were observed in *A. thaliana* in response to cinnamon essential oil (Ben Kaab et al., 2024). Perturbation of protein biosynthesis can be caused by DNA and RNA damage (transcription apparatus) (Choudhury et al., 2017), ribosomes (Salih et al., 2020), as well as modifying amino acids (translation apparatus) (Sahu et al., 2022), inefficient chaperone activity facilitating protein folding (Ozgun et al., 2015), and by affecting mitochondria which deliver energy for protein biosynthesis (Mansoor et al., 2023). Therefore, translation efficiency may be reduced or inhibited, or abnormal proteins may be produced. Conversely, protein damage is based on breaking peptide bonds and protein fragmentation or amino acids oxidation, leading to carbonyl groups or disulfide bridges formation in inappropriate places, which triggers protein misfolding or aggregation and, eventually, their denaturation and biological activity loss (Rinalducci et al., 2008). Protein structure modifications triggered by ROS make them easier to identify by cellular protein quality control system directing them for priority degradation in proteasomes (Sun et al., 2021). In our studies, we observed a tendency towards a decrease of protein content in Fabaceae (where the significant H_2O_2 accumulation occurred – about 350 % of control) although this change was not statistically significant in *V. faba*, it was distinct in *L. luteus*. In Brassicaceae, where H_2O_2 accumulation did not exceed 200 % of control, we observed an opposite trend – a protein content increase, though statistically significant changes occurred only in *B. napus*. In this case, an increase in H_2O_2 content can be recognized as moderate oxidative stress, letting highly metabolically active root cells synthesize defense proteins rather than accumulating damaged ones due to an overwhelmed proteasome system (Kosová et al., 2018). In the second case, cells may activate other mechanisms, such as autophagy, to eliminate accumulated protein aggregates and restore homeostasis (Petersen et al., 2024) which should be verified in the latter studies.

Both SEO components and their oxidative stress generated by cellular H_2O_2 accumulation could indirectly (by impacting protein content) or directly change sugar management. Generally, in the first phases of oxidative stress or in mild stress conditions, a transient accumulation of monosaccharides (reducing sugars) can occur before their depletion (Dong et al., 2023; Saddhe et al., 2021). In the case of the current study, both an increase (*L. luteus*, *A. thaliana*) and a decrease (*V. faba*, *B. napus*) in monosaccharide content were observed in Fabaceae and Brassicaceae roots making it challenging to discern similar tendencies or reactions among plants belonging to the same family in response to SEO. A similar accumulation of soluble sugars was observed in *A. thaliana* seedlings treated with *Origanum vulgare* L. essential oil (Araniti et al., 2018b) and in *E. crus-galli* after *Mentha* $\times$ *piperita* L. essential oil treatment (Synowiec et al., 2019). Conversely, decreased sugar content occurred in farnesene-treated *A. thaliana* seedlings

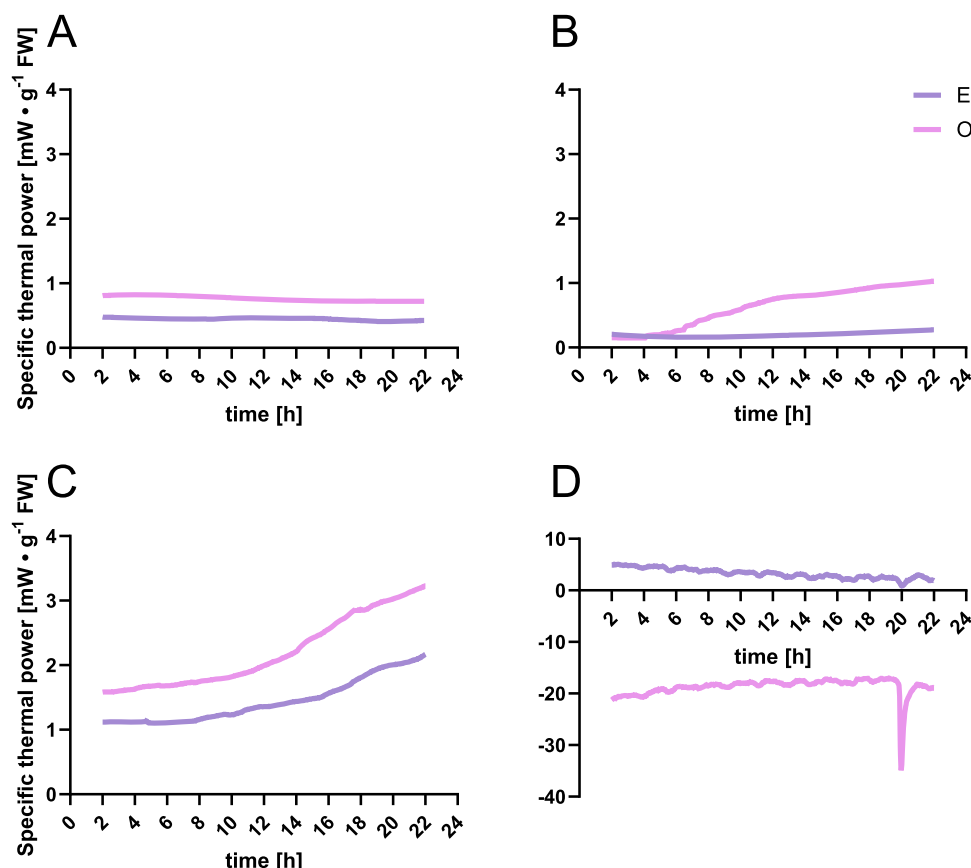


Fig. 12. The specific thermal power curves of A) *V. faba*; B) *L. luteus*; C) *B. napus*; D) *A. thaliana* seedlings during 24-hour incubation with emulsifier – E and essential oil – O.

(López-González et al., 2023) as well as after rosmarinic acid treatment due to a decrease in photosynthetic efficiency (Araniti et al., 2018a). Fluctuations in monosaccharide content can be related to the interference of stress factors with carbohydrate metabolism enzymes. Stress can both lead to the mobilization of reserves (starch and sucrose) for delivering energy for defense mechanisms and affect the activity of enzymes such as amylase (responsible for starch hydrolysis) or invertase (hydrolyzing sucrose to glucose and fructose). The accumulation of sugars under stress may result from their osmoprotective role in maintaining osmotic balance (Saddhe et al., 2021), while their decrease may be due to increased consumption, for instance, during the production of antioxidants (such as ascorbate) or enhanced energy production through glycolysis and the Krebs cycle. The changes in monosaccharide levels observed in the current study did not directly correlate with fluctuations in protein content or non-enzymatic antioxidants. ROS could also lead to perturbations of the transport of reserve sugar hydrolysates from cotyledons to growing primary roots and contribute to sugar degradation, resulting from their fragmentation or oxidation, eliminating them from cellular metabolism. Moreover, monosaccharides that are precursors to the polysaccharides building cell walls could be mobilized during the adaptive phase of stress (Polko and Kieber, 2019). Conducted research confirmed the impact of SEO on the sugar metabolism of the tested seedlings. However, in this step, it is hard to determine which pathway of the sugar metabolism was affected by both the SEO and the accumulating H₂O₂. It should be noted that changes in monosaccharide content in cells are quite dynamic. In seedlings of different species, these changes may have occurred over slightly different time frames. Although the trend in their content change could have been similar, it was not captured due to a single measurement point (after 24 hours of

incubation).

Fatty acids are the main components of membrane lipids. Aside from their impact on maintaining membrane fluidity, fatty acids serve as energy reserves, precursors for biomolecule biosynthesis, and even as signaling molecules (He et al., 2020). They can be classified into 3 groups differing in the number of double bonds determining the level of desaturation: Saturated Fatty Acids (SFA), Monounsaturated and Polyunsaturated Fatty Acids (MUFA and PUFA, respectively) (Staszak et al., 2023). The composition and proportions change in response to stress, allowing membrane function and cell integrity to be maintained. Saturated fatty acids decrease fluidity and increase the rigidity of cell membranes, as they may protect plants during high temperatures (Zoong Lwe et al., 2021). Unsaturated fatty acids increase membrane fluidity, which is particularly important during low temperatures (Liu et al., 2022). Alterations in fatty acid composition facilitate cell membrane adaptation to stress conditions such as oxidative stress (Zemanová et al., 2015). In our studies, SEO-treated seedlings showed overall elevated levels of H₂O₂, and a significant change in fatty acids profile manifested by increased amounts of SFA (excluding *L. luteus* in the last parameter). Listed parameters were notably higher in Brassicaceae and *V. faba*, so signaling and metabolic pathways leading in three species to increased SFA biosynthesis to stabilize membranes and decrease their permeability were triggered. On the other hand, the increase in SFA could be related to the inactivation of desaturases responsible for SFA transformation into MUFA and PUFA. An above-mentioned adverse reaction of *L. luteus* seedlings on oxidative stress, manifesting in SFA decrease with simultaneous increase of PUFA may result from increased carotenoid levels (preventing ROS spreading in lipid membranes (Swapnil et al., 2021)) or, on the other hand, from affected fatty acids

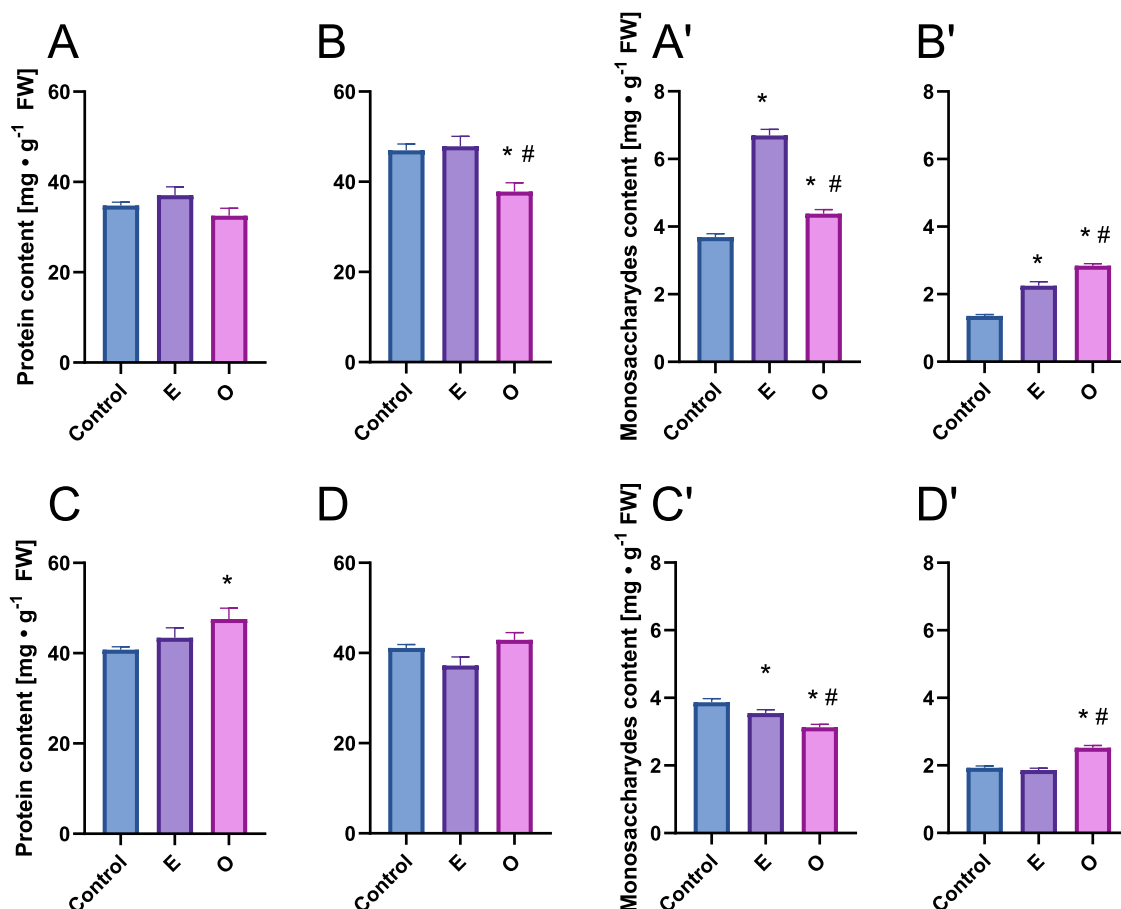


Fig. 13. Total protein (A–D) and monosaccharides content (A'–D') in primary roots of A, A') *V. faba*; B, B') *L. luteus*; C, C') *B. napus*; D, D') *A. thaliana* after 24-hour treatment with water – Control, emulsifier – E, and essential oil – O at IC₅₀ concentration. Data are presented as means ± SEM from four biological replicates. Statistical differences were assessed using Student's *t*-test ($p \leq 0.05$). Asterisk (*) and hash (#) indicate significant differences compared to the control and the emulsifier treatments, respectively.

synthesis process by ROS (Yang et al., 2022). The increased levels of unsaturated fatty acids, even under oxidative stress conditions as observed in *A. thaliana*, may result from disturbances in cellular signaling (Liang et al., 2023) or severe membrane damage forcing cells to engage in intensive repair processes that require the synthesis of both types of fatty acids. Unsaturated fatty acids may be peroxidized, which affects the membranes and, parallelly, is a signal for stress response (He et al., 2020; Somala et al., 2023). In the present studies, despite the antioxidative mechanism stimulation, accumulating ROS species led to enhanced lipid peroxidation, undoubtedly affecting the fluidity of cell membranes. The phenomenon of lipid peroxidation commonly occurs in plants exposed to various stresses such as drought (Coşkun, 2023) and phytotoxic essential oils (Andriana et al., 2019; Araniti et al., 2018a; Laosinwattana et al., 2018; Pouresmaeil et al., 2022, 2020; Somala et al., 2023). The degree of damage can be evaluated by the content of MDA, one of the final byproducts of fatty acids conversion to lipid peroxides (Zhang et al., 2005). Brassicaceae plants, due to the greater content of fatty acids, may be more vulnerable to lipid peroxidation. In line with this, the malondialdehyde (MDA) level proved to be lower (compared to control and emulsifier-treated seedlings) in Fabaceae than in Brassicaceae. The consequences of lipid peroxidation were membrane abnormalities, water loss, and electrolyte outflow manifested in increased uptake of Evans blue dye by damaged cells. Similar results were obtained for testing the bioherbicidal potential of *Hesperozygis ringens* Benth. essential oil (and its main component – pulegone) in the role of a bioherbicide towards an invasive plant *Eragrostis plana* Nees (Bruxel et al., 2022) or other essential oils originating from *Dracocephalum moldavica* L. (Pouresmaeil et al., 2022), *Eucalyptus citriodora* Hook,

Ocimum basilicum L., *Mentha arvensis* L. (Khare et al., 2019), *Piper cubeba* Bojer (Andriana et al., 2019), and *Tagetes erecta* L. (Laosinwattana et al., 2018). In our research, all the above-mentioned parameters were higher in Brassicaceae. Therefore, Fabaceae seedlings coped better with oxidative stress induced by SEO (at IC₅₀ concentration specific to each species). The reasons for such divergent responses may lie in structural factors, particularly the physicochemical properties of cell membranes, which can vary in their susceptibility to lipid peroxidation and structural damage. The lipid composition of cellular membranes differs among plant species and families. Plants with a higher β -sitosterol-to-stigmasterol ratio display greater membrane stability and reduced permeability. Such a sterol composition may limit SEO penetration into cells and reduce susceptibility to lipid peroxidation. Elevated stigmasterol levels in membrane structures have been correlated with increased permeability and vulnerability to environmental stress (Desoky et al., 2021; Dumanović et al., 2021; Grosjean et al., 2015). Investigating sterol composition in the studied plant families may help elucidate the mechanisms underlying their differential sensitivity to environmental stress.

In summary, microcalorimetric analysis combined with the evaluation of protein and monosaccharide content revealed species-specific metabolic strategies in response to SEO. In contrast, studies assessing lipid peroxidation – resulting from an altered ratio of saturated (SFA) to mono- and polyunsaturated fatty acids (MUFA and PUFA) – indicated greater susceptibility of Brassicaceae plants to SEO. A closer look at the intrinsic physiological traits of seeds and early seedlings provides additional context for interpreting these species-specific responses to SEO. The species selected for this study are characterized by diverse seed

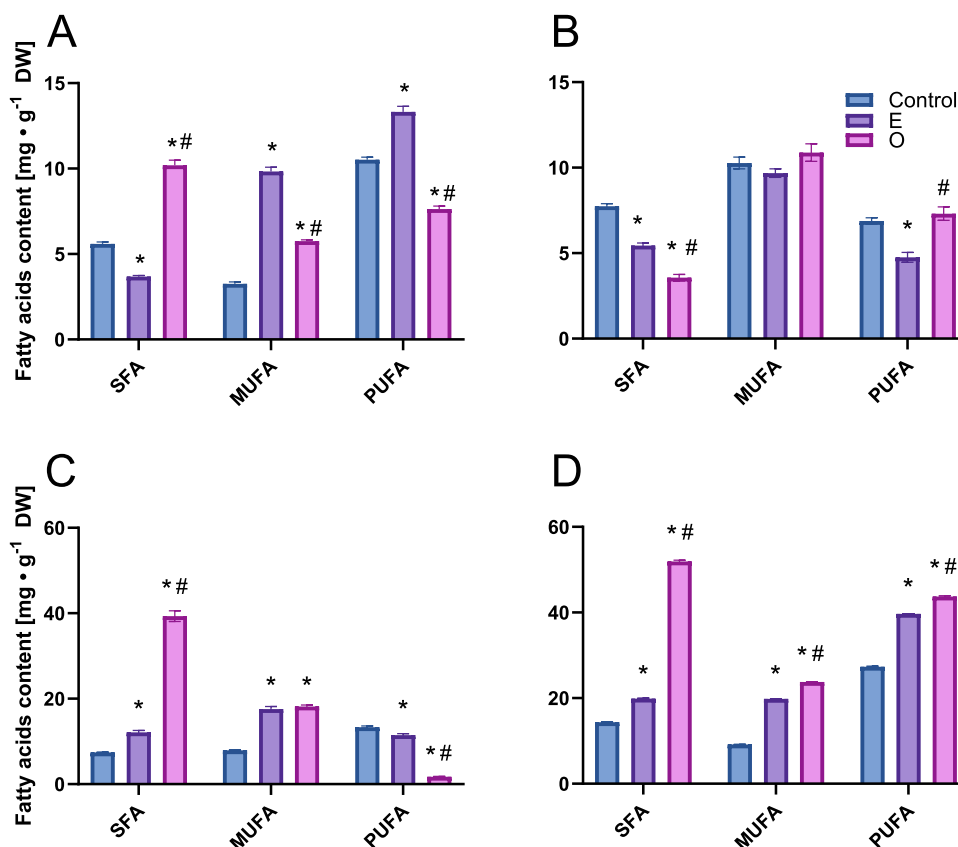


Fig. 14. Saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids content in primary roots of A) *V. faba*; B) *L. luteus*; C) *B. napus*; D) *A. thaliana* after 24-hour treatment with water – Control, emulsifier – E, and essential oil – O at IC₅₀ concentration. Data are presented as means ± SEM from three biological replicates. Statistical differences were assessed using Student's *t*-test ($p \leq 0.05$). Asterisk (*) and hash (#) indicate significant differences compared to the control and the emulsifier treatments, respectively.

traits, differing not only in size but also in the composition of storage reserves and germination strategy (De Souza Vidigal et al., 2020; D'Hooghe et al., 2019; Navarro-Vozmediano et al., 2025; Wei et al., 2022). This indicates that physiological differences are already evident at the early stages of seedling development, with young plants utilizing distinct metabolic pathways and molecular resources. *V. faba* is a starch-rich species, also abundant in storage proteins, exhibiting a hypogeal germination type, during which the cotyledons remain below the soil surface, whereas *L. luteus* is a protein-rich species with a low lipid content and insignificant starch reserves (Kokten et al., 2022; Spina et al., 2022). In contrast, *B. napus* and *A. thaliana* are classified as oilseed species, characterized by high lipid content and moderate levels of storage proteins (Karydogianni et al., 2023; Kelly, 2018). Apart from *V. faba*, these species exhibit epigeal germination, in which the cotyledons are raised above the soil surface and rapidly undergo greening (Wulf et al., 2020). The variation in dominant storage reserves and germination strategy, which determines the seedling development rate and the activation of different metabolic pathways, may influence the preferred energy acquisition mechanisms in young roots and modulate their response to stress induced by SEO application. In starch- and protein-storing species, greater membrane integrity may result from the prevalence of energy acquisition pathways based on carbohydrate or protein metabolism, which are associated with lower reactive oxygen species production compared to the intense lipid β -oxidation (Borek et al., 2015; Kuryata et al., 2020). Conversely, in oilseed species, where early seedling development relies heavily on the mobilization of triacylglycerols through β -oxidation in peroxisomes, higher ROS production was observed, leading to increased lipid peroxidation and more extensive membrane damage (Eastmond, 2006; Graham, 2008). The

obtained results indicate that the type of dominant storage reserves in seeds and their mobilization pathways during early development play a crucial role in shaping differences in the susceptibility of young plants to oxidative stress induced by SEO.

5. Conclusions

There is an urgent need to identify and thoroughly evaluate plant-derived secondary metabolites that could reduce or even replace synthetic chemical pesticides. *Acorus calamus* essential oil (SEO), known for its medicinal properties, demonstrates promising phytotoxic effects and potential application in sustainable agriculture. In the present study, SEO applied at species-specific IC₅₀ concentrations inhibited root growth and triggered oxidative stress in all tested seedlings, with distinct responses at the cellular level. These findings underscore both species- and family-specific reactions, suggesting a complex mode of action involving redox regulation and membrane destabilization.

Although the study included only a limited number of representatives from each family, certain trends were evident. Fabaceae species exhibited greater tolerance to oxidative stress induced by SEO, with milder membrane damage and higher viability, whereas Brassicaceae species appeared more susceptible to lipid peroxidation and showed more pronounced cellular and biochemical stress responses under equivalent growth-inhibitory conditions. These differences suggest that SEO concentrations effective in controlling Brassicaceae weeds may exert minimal effects on economically important leguminous crops, such as those from the Fabaceae family.

This desired selectivity offers a foundation for the development of targeted and environmentally safe weed management strategies. Unlike

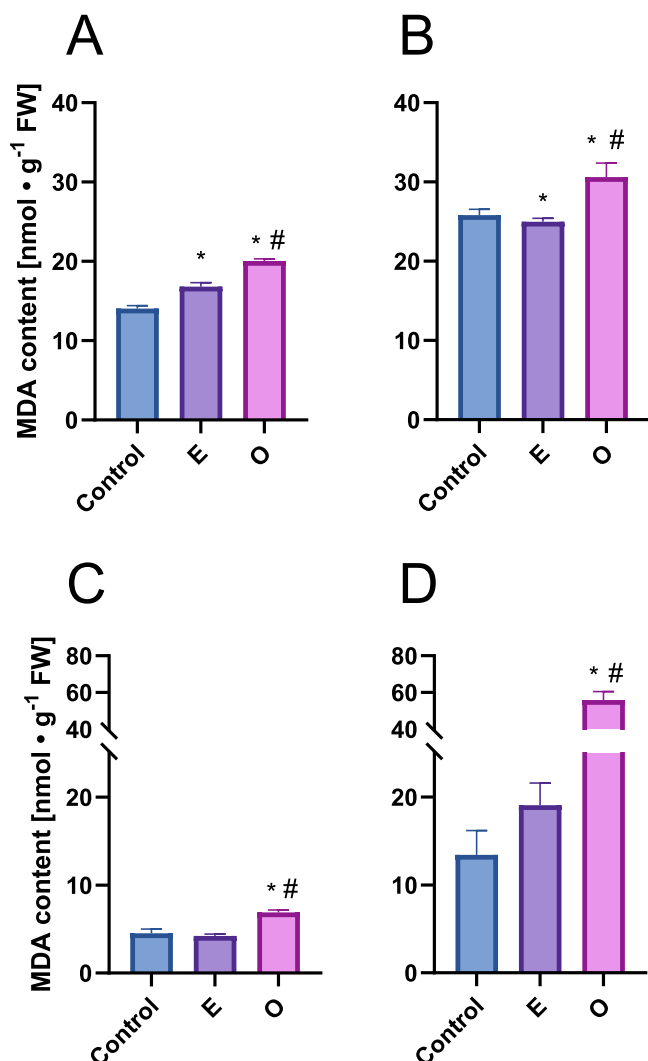


Fig. 15. Level of lipid peroxidation in roots of A) *V. faba*; B) *L. luteus*; C) *B. napus*; D) *A. thaliana* after 24-hour treatment with water – Control, emulsifier – E, and essential oil – O at IC₅₀ concentration. Data are presented as means $\pm$ SEM from four biological replicates. Statistical differences were assessed using Student's *t*-test ($p \leq 0.05$). Asterisk (*) and hash (#) indicate significant differences compared to the control and the emulsifier treatments, respectively.

synthetic herbicides that often act through single molecular targets, SEO affects multiple metabolic pathways, including ROS production, antioxidant responses, and changes in sugar and lipid profiles. This systemic mode of action reduces the likelihood of rapid resistance development in weed populations. Moreover, due to its natural origin and biodegradability, SEO minimizes chemical load in agroecosystems and aligns with the principles of sustainable agriculture.

The results presented here provide strong support for the further investigation of SEO and other allelochemicals as effective bio-herbicides. Their potential to reduce synthetic pesticide usage not only contributes to environmental conservation but also offers economic benefits, particularly when integrated into sustainable crop protection strategies. However, before broader implementation, additional research is necessary. This should include studies on a wider range of crop and weed species, as well as in-depth analyses of SEO's effects on cellular processes such as the cell cycle (including DNA replication and mitosis), and gene expression, which remain largely unexplored. In the subsequent stages of our work, we have already undertaken such studies.

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CRediT authorship contribution statement

Mateusz Wróblewski: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Alicja Piotrowska-Niczyporuk:** Writing – review & editing, Validation, Resources, Methodology, Investigation. **Iwona Ciereszko:** Writing – review & editing, Validation, Methodology. **Natalia Goczek:** Visualization. **Aneta Żabka:** Resources. **Paweł Szczepkowski:** Resources. **Łukasz Sobiech:** Resources. **Diana Saja-Garbarz:** Methodology. **Justyna T. Polit:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.scienta.2025.114180](https://doi.org/10.1016/j.scienta.2025.114180).

Data availability

Data will be made available on request.

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Article

Acorus calamus L. Essential Oil Induces Oxidative Stress and DNA Replication Disruptions in Root Meristem Cells of Two Fabaceae and Two Brassicaceae Species

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Abstract: Environmental concerns regarding synthetic herbicides have sparked interest in plant-derived bioactive compounds as eco-friendly alternatives. This study investigated the cellular targets of sweet flag essential oil (*Acorus calamus* L., SEO at IC₅₀ concentration) in root meristem cells of Fabaceae (*Vicia faba*, *Lupinus luteus*) and Brassicaceae (*Brassica napus*, *Arabidopsis thaliana*), focusing on reactive oxygen species (ROS) accumulation (DAB, NBT staining), DNA replication dynamics (EdU labeling), and genome integrity (γ-H2AX immunocytochemistry, TUNEL assay, and DNA electrophoresis). SEO induced oxidative stress (200–250% of control depending on the species) and replication stress, causing DNA double-strand breaks in 50% of proliferating cells, confirmed by γ-H2AX/TUNEL. Consequently, cells were prolonged in the G₁ phase, replication activity dropped to 70% of control in Fabaceae and 80% in Brassicaceae, and EdU incorporation intensity decreased to 80% and 70% of control, respectively. An increased proportion of cells replicating heterochromatin indicated slowed S-phase progression. Despite genotoxic effects, SEO did not trigger endoreplication, apoptotic DNA fragmentation, or extensive cell death. All species exhibited a uniform stress response, although sensitivity varied, which previously enabled the establishment of selective SEO doses between Fabaceae and Brassicaceae. These findings suggest that SEO exerts phytotoxicity by disrupting S-phase progression, supporting its potential as a selective bioherbicide.

Keywords: bioactive plant compounds; bioherbicide; cell cycle; DNA-strand breaks; γ-H2AX phosphorylation; TUNEL assay



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1. Introduction

Environmental pollution caused by chemical pesticides is related to the intensification of agriculture, which aims to produce abundant and high-quality crop yields. Irrational use of pesticides contributes to their loss of function, increasing pest resistance, and, consequently, exceeding the determined doses and withdrawal of the plant protection product. The chemicals used are often accumulated in soil and crops, endangering the health of consumers [1–3]. These problems drive to search for efficient, biodegradable, natural compounds that neither accumulate in soil nor contaminate water and show no toxicity towards people and animals. Plant secondary metabolism products, including a huge variety of essential oils, seem to be a promising solution to the abovementioned problems [4]. The selective and diversified impact of essential oils on the germination and seedling

development of several species brings attention to their potential use as biostimulators or bioherbicides [5]. A particularly important target for the action of bioactive compounds, including essential oils, is the cell cycle, as it is highly sensitive to environmental and chemical stress factors [6–8]. Moreover, proliferation, starting from the stage of germination and the formation of the primary root, directly determines plant growth. Inhibiting the cell cycle is therefore an effective strategy for eliminating unwanted weeds. Bioactive compounds can affect the cell cycle either directly or indirectly by inducing excessive production of reactive oxygen species (ROS), leading to oxidative stress [9]. This shift in balance toward harmful oxidation reactions results in damage to the structure and function of DNA, proteins, and lipids [10]. ROS pose a threat, not only to the structure of genetic material and the mitotic apparatus, but also to the function of molecular regulatory factors that form the basis of the action of cell cycle checkpoints, located at critical transitions between the G1/S and G2/M phases, as well as during key processes such as DNA replication and mitotic chromosome segregation [11].

The key regulators of the plant cell cycle are complexes of cyclin-dependent kinases (CDKs: CDKA, CDKB1, and CDKB2) with cyclins (Cyc A, B, D, and E). CDKs differ in their expression timing during the cell cycle and their functional specificity [12]. CDKA is present throughout the entire cycle, whereas CDKB1 and CDKB2 are primarily expressed from the S to M phase [13]. Cyclins are regulatory proteins whose concentrations fluctuate depending on the cell cycle phase. The activity of CDK-cyclin complexes is regulated both through phosphorylation/dephosphorylation processes and by interaction with CDK inhibitors (CKIs) belonging to the Kip-Related Protein (KRP) family [14]. In response to external signals, such as hormones and nutrient availability, cyclin D synthesis is induced. By binding to CDKA, cyclin D promotes the expression of cyclin E, another CDKA partner. Active CDKA-CycA and CDKA-CycE complexes phosphorylate the Rb protein, leading to the release of the transcription factor E2F, which activates genes involved in DNA replication [15]. This marks the transition of the cell into the S phase, during which a sophisticated and tightly regulated machinery ensures DNA synthesis, allowing the parent cell to transmit an exact genetic copy to the two daughter cells. This process occurs simultaneously at multiple sites within the genome and is organized through the existence of replicons—replicative units that include the origin of replication, replication forks that branch out in opposite directions, and the replication termination sites. The origin sites are gradually activated during the S phase. Replicons can be classified as early or late, depending on when they are activated within the S phase. Early replicons are initiated at the beginning of the S phase and typically encompass actively transcribed euchromatin regions, while late replicons replicate heterochromatic areas of the genome at later stages. In the classical cell cycle, each replicon, regardless of activation timing, is only replicated once during each S phase [16].

DNA synthesis requires the coordinated action of multiple enzymes and stabilizing proteins. Helicase unwinds the DNA by breaking hydrogen bonds, allowing access to the template strand. Single-stranded DNA binding proteins (SSBs) prevent re-annealing and degradation, stabilizing the unwound DNA. Topoisomerase, ahead of the replication fork, alleviates supercoiling by cutting and rejoining DNA strands. Primase synthesizes short RNA primers, initiating replication for DNA polymerases. DNA polymerase α facilitates initiation, while δ and ϵ elongate and proofread the lagging and leading strands, respectively, adding nucleotides in a 5' $\rightarrow$ 3' direction. Exonucleases remove RNA primers and excise replication errors. DNA ligase seals Okazaki fragments, ensuring strand continuity [6].

To maintain genome stability, the complex process of genetic material duplication is tightly regulated by a sophisticated system of molecular mechanisms that enable cells to detect, signal, and repair DNA damage in a process known as DNA Damage Response

(DDR) [12]. The detection and identification of DNA damage are carried out by specialized sensors: the MRN complex (MRE11-RAD50-NBS1), which detects double-strand breaks (DSBs) and recruits the ATM kinase (Ataxia-Telangiectasia Mutated); RPA (Replication Protein A), a protein that recognizes single-stranded DNA (ssDNA) and stabilizes the replication fork; and the 9-1-1 complex (RAD9-RAD1-HUS1), which functions as a ring that encircles DNA at single- to double-strand junctions and activates ATR kinases (Ataxia Telangiectasia Mutated and Rad3-related). Activated by both single- and double-strand DNA breaks, as well as by replication fork slowing or stalling, ATM and ATR kinases initiate the response. This results in the phosphorylation of histone H2A.X, leading to the recruitment of repair proteins to the damage sites and activation of the repair pathway [12,17]. Phosphorylation also occurs in cell cycle regulatory proteins, including the transcription factor protein SOG1 (Suppressor of Gamma Response 1), a functional equivalent of the p53 protein in animal cells but with no sequence homology to it [18], which induces the expression of cyclin inhibitors and genes associated with DNA repair or programmed cell death if the damage is too severe. Additionally, the WEE1 kinase is phosphorylated, which inhibits the activity of CDK/Cyclin complexes, thus halting the cell cycle at the S or G2/M phase and allowing time for the activation of repair mechanisms and DNA repair [12]. If the damage is too extensive to repair, DDR can trigger programmed cell death (PCD, apoptosis-like death) or induce cell senescence. Regulation of the S phase in plant cells combines mechanisms characteristic of other eukaryotes with unique adaptations that enable plant cells to respond to changing environmental conditions, such as nutrient availability and abiotic stress. This highlights the plasticity of the cell cycle in plants. Under unfavorable conditions, plants can slow down or even halt replication to protect cells from duplicating damaged DNA [17].

This study builds on previous research on SEO composition and its biological activity by examining its impact on plant root growth. The major compounds identified in SEO include phenylpropanoids (β -, α -, and γ -asarone), monoterpenes (α - and β -pinene, camphene, myrcene, and limonene), sesquiterpenes (cadinene, acorone, acorenone, and calamine), tannins, choline, and fatty acids [19]. Despite extensive studies on SEO, its specific effects on plant development and cellular responses remain insufficiently understood. This study examined the primary roots of two Fabaceae species (*Vicia faba* var. *minor* L. and *Lupinus luteus* L.) and two Brassicaceae species (*Brassica napus* L. and *Arabidopsis thaliana* L.) which were incubated for 24 h in sweet flag (*Acorus calamus* L.) essential oil (SEO) at an IC_{50} concentration—individually determined for each species—that inhibited root growth by 50% [20]. To test our hypothesis that *Acorus calamus* L. essential oil (SEO) inhibits root growth by inducing oxidative stress that disrupts DNA replication during the S phase of the cell cycle, we analyzed reactive oxygen species (ROS) accumulation, assessed DNA replication dynamics, and examined DNA integrity in root meristems. We further hypothesized that these disruptions might differ between species, revealing either species-specific responses, similarities within plant families, or broader distinctions between Fabaceae and Brassicaceae.

2. Results

2.1. The Impact of SEO on H_2O_2 and $O_2^{\bullet-}$ in Root Meristematic Cells

One of the most commonly used methods to visualize the location of hydrogen peroxide (H_2O_2) is based on the oxidation of diaminobenzidine (DAB) by H_2O_2 produced in cells. The oxidized DAB appears as brown precipitates visible under a light microscope (Figure 1A–D"). In the roots of seedlings from all four studied species—previously incubated for 24 h in water (control), emulsifier, and emulsified SEO at an IC_{50} concentration—meristems treated with the emulsifier exhibited a low level of staining, similar to that of

the control (Figure 1A–D',E–H). In contrast, the most intense staining was observed in the root meristem zones treated with SEO, particularly in *L. luteus* and *A. thaliana*, where the staining reached approximately 250% of the control level (Figure 1B'',D'',F,H), and to a slightly lesser extent in *V. faba* and *B. napus*, where it was around 200% of the control (Figure 1A'',C'',E,G). Microscopic analyses revealed both the dispersed cytoplasmic location of DAB precipitates, as well as brownish deposits clearly grouped in oval structures primarily surrounding the cell nucleus, which always remained brighter than the other areas of the cell (Figure 1A''–D'').

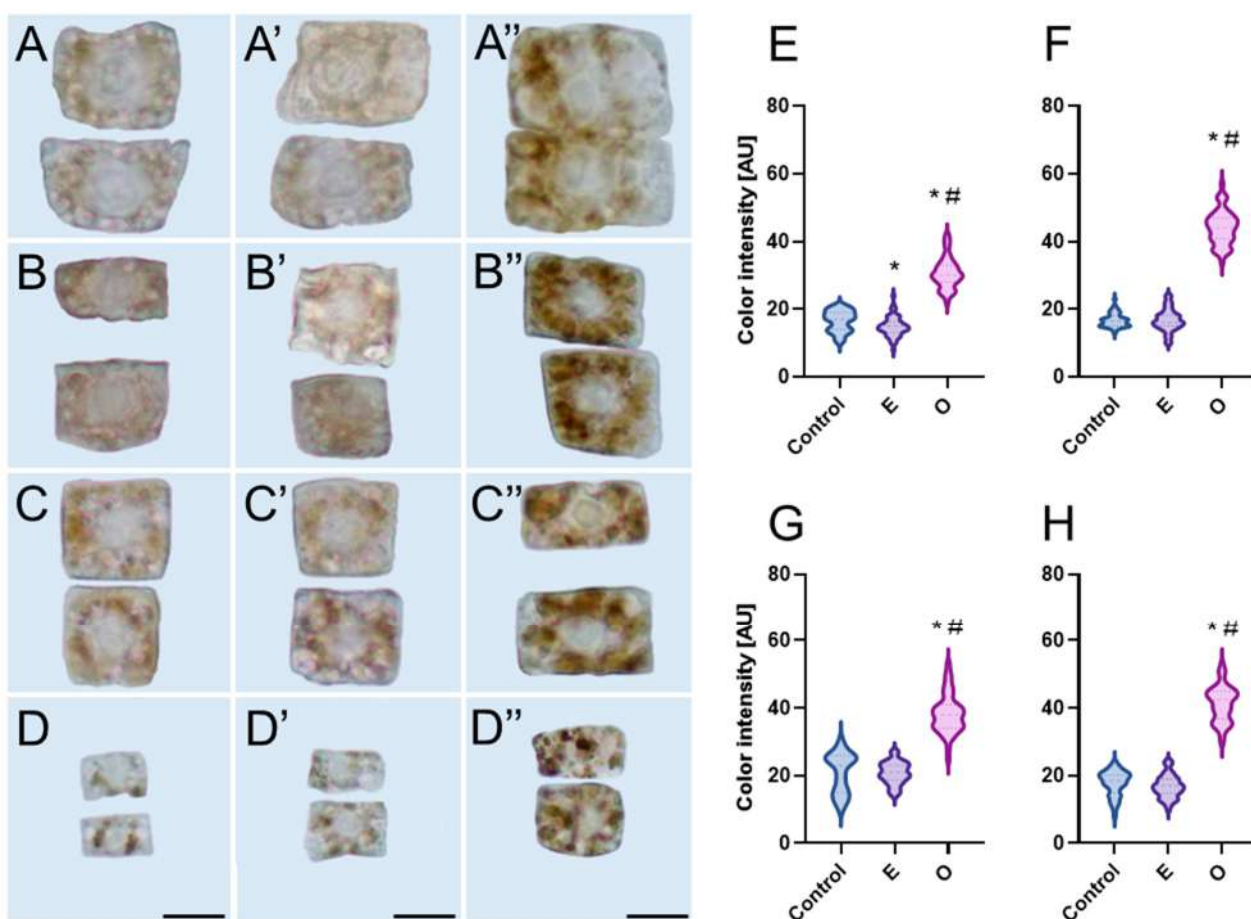


Figure 1. Generation of H_2O_2 visualized as brown deposits of oxidized diaminobenzidine (DAB) and corresponding cytometric evaluation of mean DAB staining intensity [arbitrary units, AU] in *Vicia faba* (A–A'',E), *Lupinus luteus* (B–B'',F), *Brassica napus* (C–C'',G), and *Arabidopsis thaliana* (D–D'',H) root meristem cells (N = 50) after 24h seedling incubation in water—Control (A–D), the emulsifier solution—E (A'–D'), or emulsified SEO at the IC₅₀ concentration—O (A''–D''). Scale bar: 10 μ m. The width of each violin plot represents the distribution of values along the y-axis; medians are indicated by dashed lines and quartiles by dotted lines. Statistical differences were assessed using Welch's *t*-test and the Mann–Whitney test at $p \leq 0.05$. Asterisk (*) and hash (#) marks denote significant differences compared to the control and the emulsifier, respectively.

The level of the second type of ROS analyzed, the superoxide anion radical ($O_2^{\bullet-}$), was assessed using nitroblue tetrazolium (NBT) reduction. The yellow NBT solution enters the cells, where it is reduced in the presence of $O_2^{\bullet-}$ to a blue, water-insoluble formazan, visible under a light microscope as precipitates (Figure 2A–B''). The reaction was conducted only on representatives of the Fabaceae and Brassicaceae families (*V. faba* and *B. napus*) in which a slightly lower H_2O_2 content was recorded. Similar to the identification of H_2O_2 , a significant amount of $O_2^{\bullet-}$ was detected in the root meristematic zone treated with SEO,

compared to the control and emulsifier-treated meristems. In the microscopic images, dark blue formazan deposits formed fine granules scattered throughout the cytoplasm and concentrated near the cell nuclei (Figure 2A–B''). Despite the similarities in H_2O_2 content in the root meristematic cells of both species treated with SEO, the quantitative analysis of formazan deposits in individual cells, based on staining intensity, indirectly indicated a significantly higher level of $\text{O}_2^{\bullet-}$ in the root meristems of *V. faba* compared to *B. napus*, while the assessment of staining intensity increase in SEO-treated cells relative to the control revealed an $\text{O}_2^{\bullet-}$ content rise to 200% in *V. faba* and up to 275% in *B. napus* (Figure 2C,D).

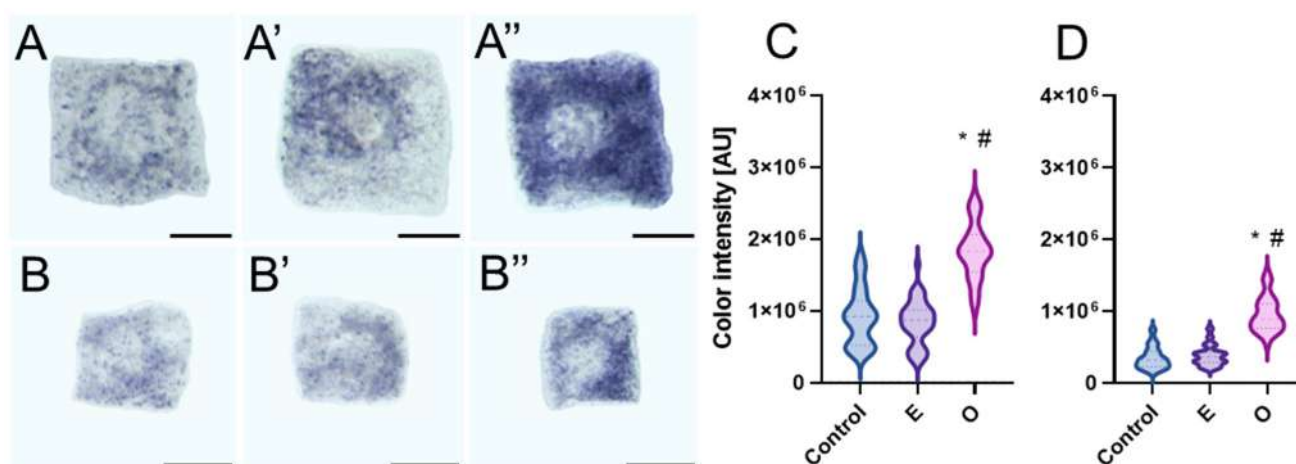


Figure 2. Generation of $\text{O}_2^{\bullet-}$ detected by NBT staining and corresponding cytometric evaluation of mean NBT staining intensity [arbitrary units- AU] in *V. faba* (A–A'',C) and *B. napus* (B–B'',D) root meristem cells (N = 50) after 24 h of seedling incubation in water—Control (A,B), the emulsifier solution—E (A',B'), or emulsified SEO at the IC_{50} concentration—O (A'',B''). Scale bar: 10 μm . The width of each violin plot represents the distribution of values along the y-axis; medians are indicated by dashed lines and quartiles by dotted lines. Statistical differences were assessed using the Mann–Whitney test at $p \leq 0.05$. Asterisk (*) and hash (#) marks denote significant differences compared to the control and the emulsifier, respectively.

2.2. Assessment of the Replication Process and the Percentage Distribution of Meristematic Cell Populations Based on DNA Content

The reduction of root growth to half of the control value under the influence of SEO, accompanied by an elevated ROS level in the meristematic zone, may result from disturbances in the cell cycle, where DNA replication during the S phase is a critical yet highly oxidative stress-sensitive stage. The first signs of these irregularities appear as disruptions in the distribution of cells across different interphase stages.

The primary methods for identifying cells in the G1, S, and G2 phases include cytophotometric assessment of DNA content in Feulgen-stained nuclei (Figure 3) and fluorescent detection of 5-ethynyl-2'-deoxyuridine (EdU), a thymidine analog that incorporates into newly synthesized DNA strands, thereby labeling the nuclei of replicating cells (Figure 4), and thus allowing the calculation of their percentage (labeling index, Figure 5).

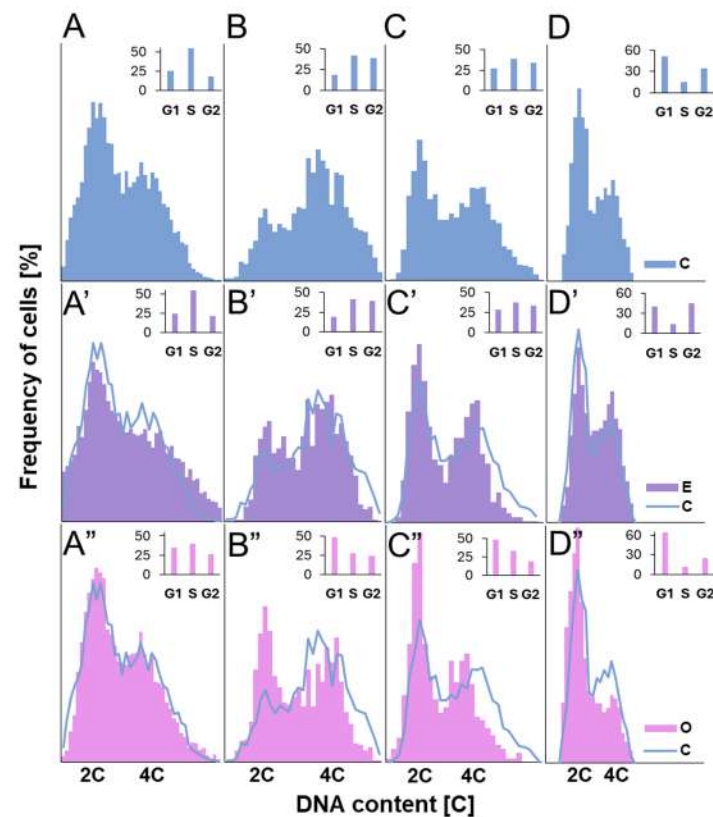


Figure 3. Frequency distribution [%] of nuclear DNA content [C] in root apical meristem cells of *V. faba* (A–A''), *L. luteus* (B–B''), *B. napus* (C–C''), and *A. thaliana* (D–D'') after 24 h seedling incubation in water—C (A–D), the emulsifier solution—E (A'–D'), or emulsified SEO at the IC₅₀ concentration—O (A''–D''). Bar graphs on the right of each histogram show the percentages of cells in the G1, S, and G2 phases for the respective experimental series.

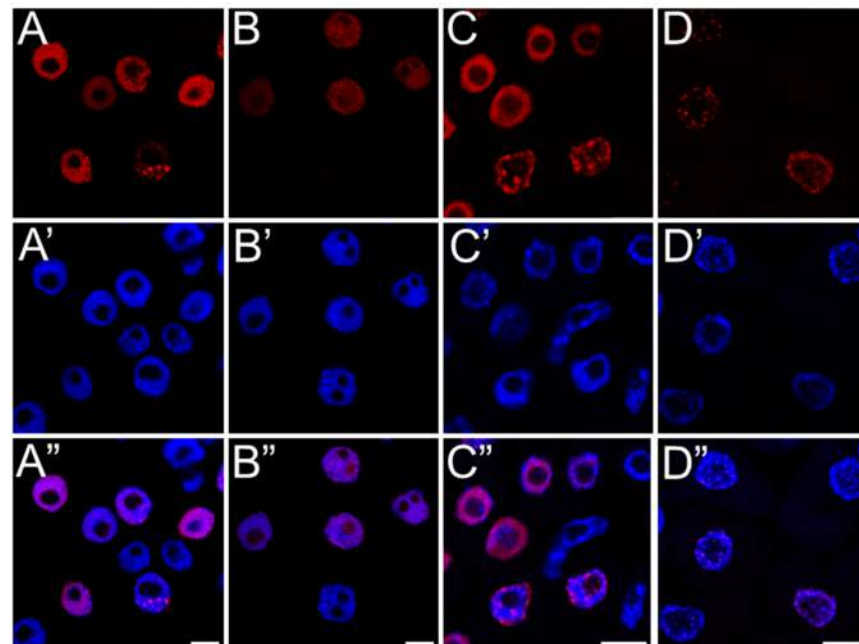


Figure 4. DNA replication detected by EdU assay in root meristem cells of *V. faba* (A,B) and *B. napus* (C,D) after 24 h seedling incubation in water (Control; (A,C)), or emulsified SEO at the IC₅₀ concentration (B,D). Corresponding DNA staining with DAPI is shown in (A'–D'), and merged images in (A''–D''). Scale bar: 10 μ m.

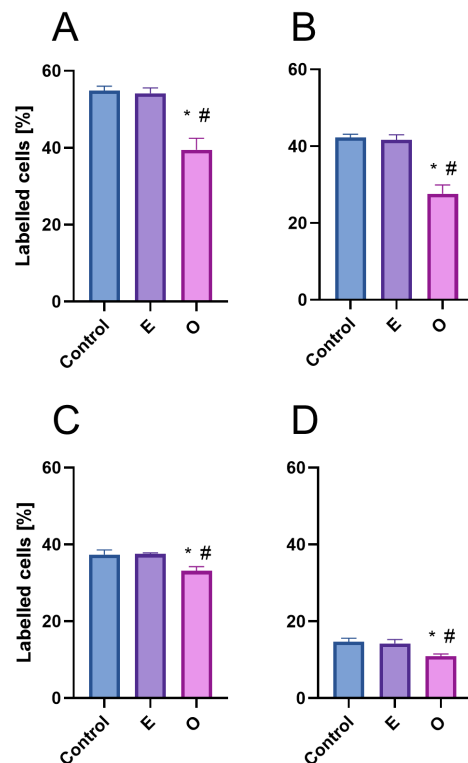


Figure 5. Labelling indices of EdU-positive root meristem cells of *V. faba* (A), *L. luteus* (B), *B. napus* (C) and *A. thaliana* (D) after 24 h seedling incubation in water—Control, the emulsifier solution—E, or emulsified SEO at the IC₅₀ concentration—O. Data are presented as mean indices $\pm$ SEM from three biological replicates. Statistical differences were assessed using Student's *t*-test at $p \leq 0.05$. Asterisk (*) and hash (#) marks denote significant differences compared to the control and the emulsifier, respectively.

The analysis revealed slight variations in population distributions among control meristems of different species and no significant changes in these distributions under the influence of the emulsifier (see Figure 3A–D' and Figure 5). The distributions were characterized by the highest proportion of cells in the S phase in *V. faba* (approximately 50%), slightly lower but similar proportions in *L. luteus* and *B. napus* (around 40%), and the lowest percentage in *A. thaliana* (15%).

Exposure to SEO at the IC₅₀ concentration caused a significant redistribution of meristematic cells across different interphase stages (Figure 3A''–D'' and Figure 5). A pronounced increase in the number of cells with 2C DNA content, characteristic of the G1 phase, was observed, accompanied by a decrease in the number of cells with 4C DNA content in the G2 phase, as well as a reduction in the population of cells with intermediate DNA content (between 2C and 4C), characteristic of the S phase. Replicating cells accounted for nearly 70% of the control level in both Fabaceae species and approximately 80% in Brassicaceae species (Figure 5).

Fluorescence intensity measurements of replicating cells revealed that SEO not only reduced the number of replicating cells but also significantly limited the incorporation of the marker into newly synthesized DNA strands (Figure 6). This reduction, characterized by weaker fluorescence intensity on the nuclear surface compared to the control, was more pronounced in Brassicaceae (70% of the control) than in Fabaceae (approximately 80% of the control; see Figure 6A–D).

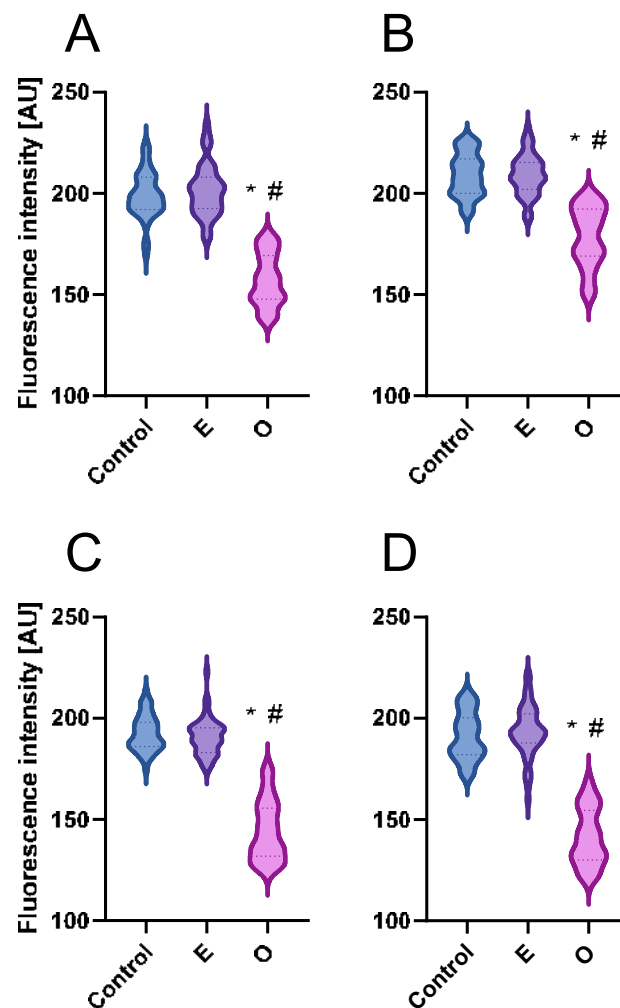


Figure 6. Mean fluorescence intensity of EdU-positive nuclei from root meristem cells in the S-phase [arbitrary units—AU] of *V. faba* (A), *L. luteus* (B), *B. napus* (C), and *A. thaliana* (D) after 24 h seedling incubation in water—Control, the emulsifier solution—E, or emulsified SEO at the IC₅₀ concentration—O. The width of each violin plot represents the distribution of values along the y-axis; medians are indicated by dashed lines and quartiles by dotted lines. Statistical differences were assessed using the Mann–Whitney test at $p \leq 0.05$. Asterisk (*) and hash (#) marks denote significant differences compared to the control and the emulsifier, respectively.

The application of a replication marker also allowed us to distinguish nuclei at three different stages of DNA replication (Figures 7 and 8) (1) an early stage. It is characterized by a small number of active replicons and, consequently, only a few fluorescent signals within the nucleus, which is similar in size to those in the G1 phase; (2) a middle stage, marked by the presence of numerous replicons and intense marker incorporation, visualized as a large number of homogeneously distributed foci occurring in nuclei that are noticeably larger than those in the early stage; and (3) a late stage, distinguished by fluorescent signals forming clusters that indicate late-replicating heterochromatin regions, in nuclei with a surface area comparable to those in the G2 phase. The analysis of the percentage distribution of nuclei at different stages of the S phase (Figure 9) clearly revealed an increase in the subpopulation of nuclei with a clustered labeling pattern, characteristic of heterochromatin replication, in the meristems of all SEO-treated species, at the expense of a decrease in the number of homogeneously labeled nuclei.

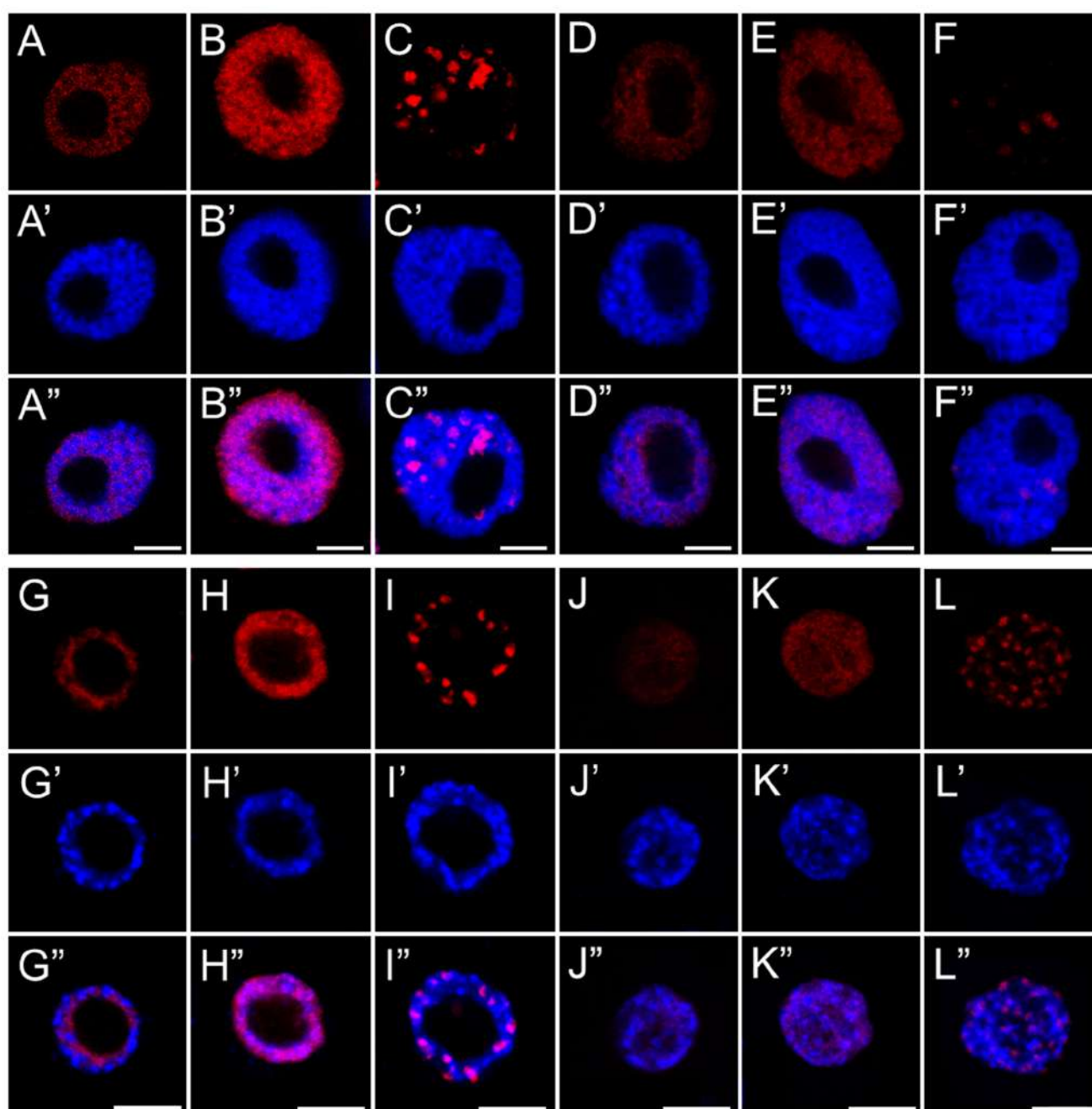


Figure 7. EdU-positive nuclei of root apical meristem cells of *V. faba* (A–F) and *L. luteus* (G–L) at three replication stages: early (A,D,G,J), mid (B,E,H,K), and late S-phase (C,F,I,L) after 24 h seedling incubation in water—Control (A–C,G–I), or emulsified SEO at IC₅₀ concentration (D–F,J–L). Corresponding DNA staining with DAPI is shown in (A'–L'), and merged images in (A''–L''). Scale bar: 5 μ m.

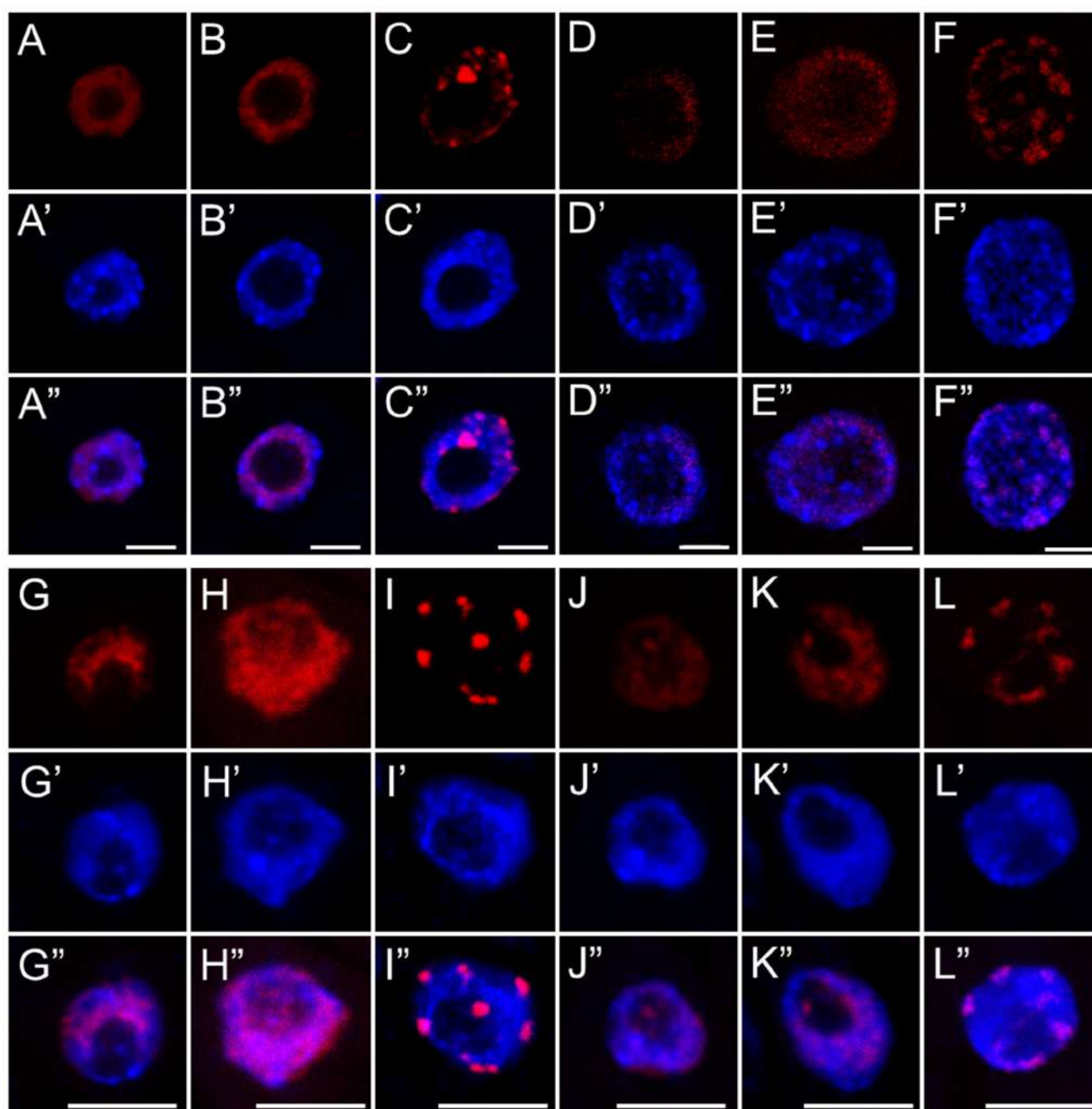


Figure 8. EdU-positive nuclei of root apical meristem cells of *B. napus* (A–F) and *A. thaliana* (G–L) at three replication stages: early (A,D,G,J), mid (B,E,H,K), and late S-phase (C,F,I,L) after 24 h seedling incubation in water—Control (A–C,G–I), or emulsified SEO at IC₅₀ concentration (D–F,J–L). Corresponding DNA staining with DAPI is shown in (A'–L'), and merged images in (A''–L''). Scale bar: 5 μ m.

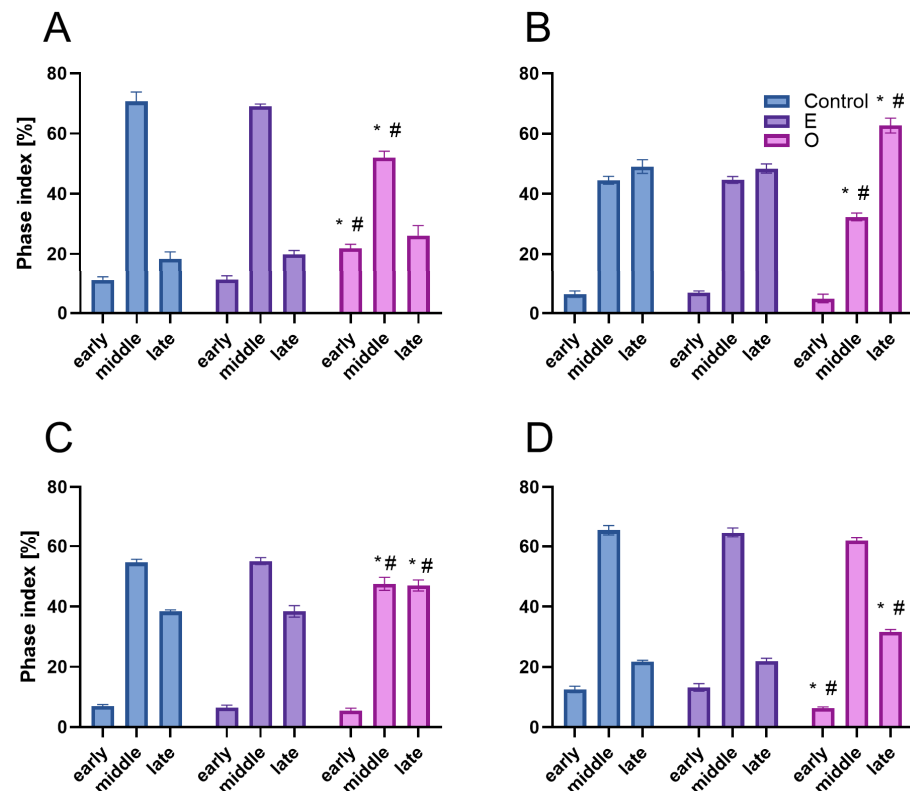


Figure 9. Fractions of nuclei [%] in root apical meristem cells of *V. faba* (A), *L. luteus* (B), *B. napus* (C), and *A. thaliana* (D) at early, mid, and late S-phase after 24 h seedling incubation in water—Control, the emulsifier solution—E, or emulsified SEO at the IC₅₀ concentration—O. Data are presented as mean indices $\pm$ SEM from three biological replicates. Statistical differences were assessed using Student's *t*-test at $p \leq 0.05$. Asterisk (*) and hash (#) marks indicate significant differences compared to the control and the emulsifier, respectively.

2.3. Evaluation of DNA Damage

Disruptions in the DNA replication process in meristems exposed to SEO may have been caused by strand integrity damage induced by excessive levels of reactive oxygen species. Therefore, immunodetection of γ -H2AX histones, a specific modification of histone H2AX that localizes to sites of double-strand breaks (DSBs) in DNA, was performed. The study was conducted on representatives of the Fabaceae (*V. faba*) and Brassicaceae (*B. napus*) families (Figure 10). Fluorescence microscopy observations revealed a weak positive signal in a few meristem cells of both the control and emulsifier-treated seedlings, indicating naturally occurring breaks during DNA replication and repair (Figures 10A,B,D,E and 11). However, after 24 h of SEO treatment, the number of labeled cells increased to approximately 50%, with clearly visible numerous fluorescence foci in the nuclear area confirming the presence of double-strand DNA breaks (Figures 10C,D and 11).

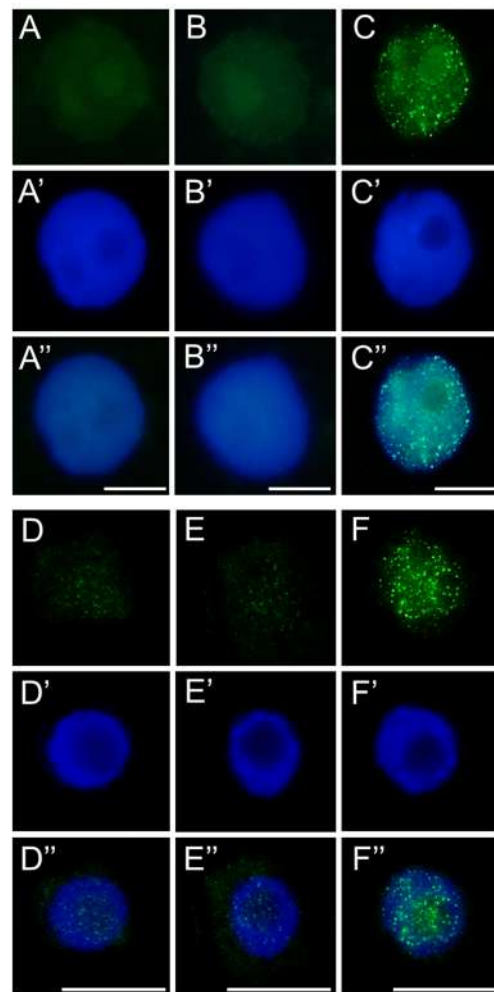


Figure 10. DNA breaks visualized by immunolabelling of γ H2AX histone modification in root meristem cells of *V. faba* (A–C) and *B. napus* (D–F) after 24 h seedling incubation in water—Control (A,D), the emulsifier solution (B,E), or emulsified SEO at the IC₅₀ concentration (C,F). Corresponding DNA staining with DAPI is shown in (A'–F'), and merged images in (A''–F''). Scale bar: 10 μ m.

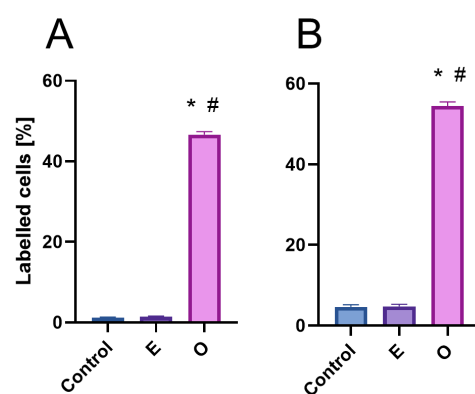


Figure 11. Frequencies of γ -H2AX positive nuclei [%] in root meristem cells of *V. faba* (A) and *B. napus* (B) after 24 h seedling incubation in water—Control, the emulsifier solution—E, or emulsified SEO at the IC₅₀ concentration—O. Data are presented as mean indices $\pm$ SEM from three biological replicates. Statistical differences were assessed using Student's *t*-test at $p \leq 0.05$. Asterisk (*) and hash (#) marks indicate significant differences compared to the control and the emulsifier, respectively.

The TUNEL method (Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling) detects both single-strand (SSB) and double-strand (DSB) DNA breaks, which may result from yet unrepaired genetic material damage as well as severe ones leading to cell death.

Terminal deoxynucleotidyl transferase (TdT), a key enzyme in the assay, incorporates dUTP nucleotides into free 3'-OH DNA ends formed during fragmentation, allowing subsequent visualization of DNA nicks via the Click-iT reaction. Microscopic observations revealed dark, non-fluorescent nuclei in control cells and weak fluorescence in the nuclei of root meristematic cells treated with SEO (compare Figure 12A–C or Figure 12E–G). Analysis of labeling indexes showed that approximately 50% of cells were positively stained after 24 h IC₅₀ SEO treatment in both tested species (Figure 13).

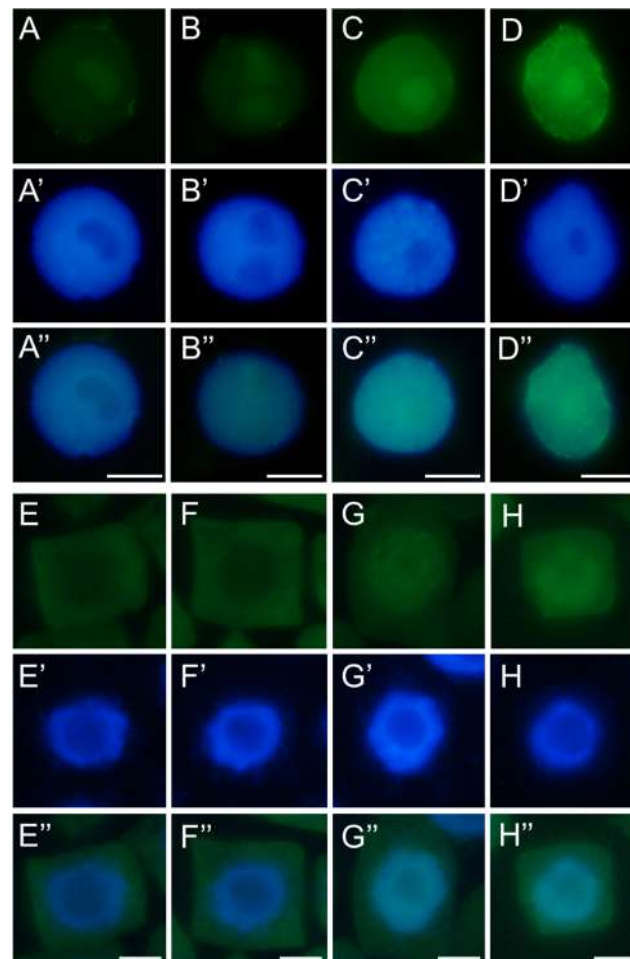


Figure 12. Detection of DNA breaks using the TUNEL assay in nuclei of root meristem cells of *V. faba* (A–D) and *B. napus* (E–H) after 24 h seedling incubation in water—Control (A,E), the emulsifier solution (B,F), or emulsified SEO at the IC₅₀ concentration (C,G). Positive control (DNase I treatment; (D,H)). Corresponding DNA staining with DAPI is shown in (A'–H'), and merged images in (A''–H''). Scale bar: 10 μ m.

DNA gel electrophoresis is a widely used method for evaluating DNA damage by separating DNA fragments in an agarose gel, resulting in either a DNA ladder pattern (more characteristic of PCD) or a smear pattern (more characteristic of necrosis). Electrophoretic separation of total genomic DNA isolated from primary roots of *V. faba* and *B. napus* treated with water, emulsifier, and SEO did not reveal DNA fragmentation in the form of DNA laddering. However, a slight smear, indicating possible DNA damage, was observed only in SEO-treated *V. faba* seedlings (Figure 14).

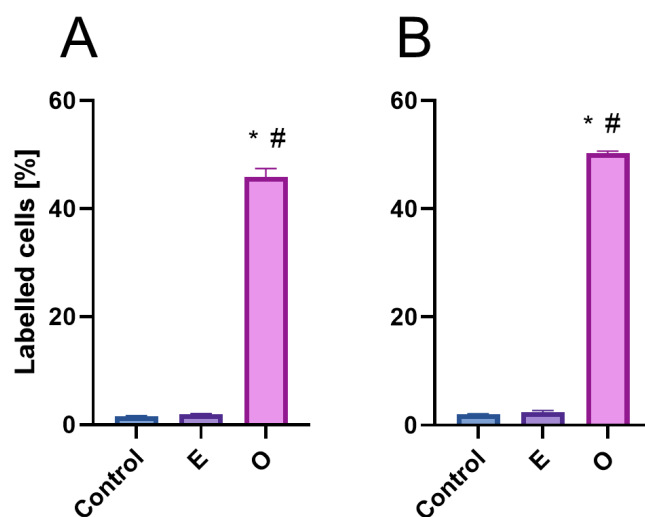


Figure 13. Frequencies of TUNEL-positive nuclei in root meristem cells of *V. faba* (A) and *B. napus* (B) after 24 h seedling incubation in water—Control, the emulsifier solution—E, or emulsified SEO at the IC₅₀ concentration—O. Data are presented as mean indices $\pm$ SEM from three biological replicates. Statistical differences were assessed using Student's *t*-test at $p \leq 0.05$. Asterisk (*) and hash (#) marks indicate significant differences compared to the control and the emulsifier, respectively.

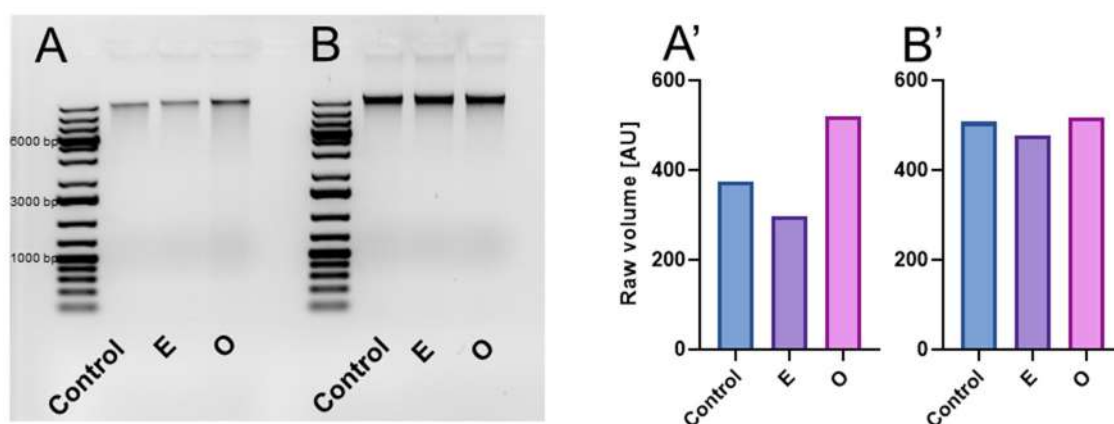


Figure 14. Gel electrophoresis of DNA (A,B) and analysis of smear volume [arbitrary units, AU] (A',B'). DNA was isolated from apical parts of *V. faba* (A,A') and *B. napus* (B,B') roots excised from seedlings after 24 h incubation in water—Control, the emulsifier solution—E, or emulsified SEO at the IC₅₀ concentration—O.

3. Discussion

In response to the growing demand for environmentally safe and effective bioherbicidal substances, recent studies have increasingly focused on the phytotoxic effects of essential oils [5,21]. This progress has been made possible thanks to the development of modern research techniques, which allow for more precise identification of the cellular targets and affected biochemical mechanisms of action of these substances in plant cells [22–25]. As a result, these studies go beyond those assessing only the general effects of essential oils on seed germination or seedling growth [26,27]. However, these efforts remain insufficient, as essential oils are complex mixtures that are difficult to evaluate unambiguously, plant species exhibit varying degrees of sensitivity to their action, and many preliminary findings still require further confirmation and elaboration [28].

Our studies focus on evaluating the bioherbicidal activity of essential oil extracted from *Acorus calamus* rhizomes (SEO) [19] against two economically important crop species (*V. faba*, *L. luteus*) from the Fabaceae family and two weed species (*B. napus*, *A. thaliana*) from

the Brassicaceae family. *V. faba* and *L. luteus* are among the most widely cultivated large-seeded legumes; however, their slow initial growth under natural conditions, combined with the limited availability of dedicated herbicides, promotes the aggressive spread of competing weeds [29,30]. Although *B. napus* is a crop species, its volunteer plants pose serious threats to legume cultivation [31]. Meanwhile, *A. thaliana* serves both as a weed and as a model organism in molecular studies. The selection of *Acorus calamus* essential oil for this study was motivated by its unique chemical composition, particularly the prevalence of phenylpropanoids such as asarones [19,26], which are known for their redox-modulating properties. Practical factors, including the oil's economic viability, the easy accessibility of *A. calamus*, and its large rhizomes, also contributed to the choice. Despite its long-standing use in traditional medicine and demonstrated bioactivity in animal models and in vitro systems [27,32,33], the molecular mechanisms of its action in plant cells remain poorly understood.

The initial stage of the study demonstrated that an appropriately selected concentration of SEO can significantly inhibit the growth of the primary roots in Brassicaceae seedlings without disrupting primary root growth in Fabaceae, and even stimulating more intensive root development in *L. luteus* [20]. Based on the IC₅₀ values determined for each species (*V. faba*—0.03%, *L. luteus*—0.025%, *B. napus*—0.01%, and *A. thaliana*—0.05%), it was possible to compare responses across taxonomic groups, investigate the cellular targets of SEO under equivalent stress intensity, and assess the potential for species- or family-specific sensitivity patterns. Analyses of oxidative stress levels and the antioxidant system response as well as general metabolic changes (via isothermal calorimetry) in primary roots confirmed the existence of distinct physiological strategies for coping with SEO exposure after 24 h incubation at IC₅₀ concentrations [20].

Primary root growth depends on both cell elongation in the elongation zone and cell proliferation occurring in the root apical meristem. While our previous study focused on general physiological and metabolic responses to SEO at the whole-organ level, the present work investigates early molecular targets within the root meristem. The accumulation of reactive oxygen species (ROS) is one of the earliest indicators of oxidative stress and can significantly influence not only antioxidant system activity and cellular metabolism but also genome stability and cell cycle progression [13,23,34]. Therefore, in this study, we visualized and quantified ROS levels in meristematic cells exposed to SEO and analyzed the progression of DNA replication during the S phase. Although root growth in the analyzed species was inhibited by 50%, ROS levels in the meristems varied, even among species within the same family, suggesting differences in stress tolerance. Variations in ROS accumulation were observed for different Fabaceae species under *Eucalyptus saligna* leaf litter treatment [35], as well in *Brassica* species under drought stress [36], which confirms our assumption.

The stress markers detected in root meristem cells accumulated mainly in cytoplasmic organelles located near the nuclei, leaving the nuclear area noticeably lighter and free of pigment deposits. This is likely due to the fact that the nucleus is not a primary site of ROS production, and even if ROS or staining reagents such as DAB reach the nucleus, the absence of peroxidases, required to catalyze the chromogenic reaction, prevents visible pigment formation. In all analyzed species, oxidized DAB (indicating H₂O₂ accumulation) was primarily localized in large organelles previously identified as plastids by electron microscopy [37,38] whereas formazan (indicating the presence of O₂^{•−}) accumulated predominantly in much smaller organelles identified as mitochondria [38]. In non-photosynthetic tissues, mitochondria are the main source of O₂^{•−}, produced during the activity of the electron transport chain. The accumulated O₂^{•−} is highly reactive and does not freely diffuse; instead, it is rapidly converted into H₂O₂ by superoxide dismutase

(SOD) [39]. What is more, ROS generation by essential oil treatment could not be the only reason responsible for organelle damage. Molecular docking studies in the course of evaluating the *Artemisia argyi* essential oil against weed species revealed the oil components exhibit binding affinity with 4-hydroxyphenylpyruvate dioxygenase enzymes. These are responsible for plastid quinones biosynthesis, and their inhibition results in affected carotenoid biosynthesis and, in turn, chloroplast dysfunction reflected in leaf bleaching and decreased pigment levels [40].

Mitochondria and plastids are highly sensitive cellular sensors of environmental changes and function as signaling hubs, maintaining bidirectional communication with the cell nucleus, which modulates gene expression in response to intracellular disturbances [41–43]. Given the presence of organelle-to-nucleus (retrograde) signaling pathways, it is plausible that, despite the absence of visible ROS marker accumulation in the nucleus, appropriate oxidative stress signals generated in the organelles were relayed to the nucleus via specific signaling cascades [43]. One well-characterized mechanism in plants involves the transcription factor ANAC017, which, in response to organelle-derived stress, is released from the ER membrane and translocated to the nucleus, where it activates the expression of stress-responsive genes [44]. Additionally, both mitochondria and plastids are capable of releasing calcium ions (Ca^{2+}) under oxidative stress. The resulting increase in cytosolic Ca^{2+} levels is detected and relayed by specific calcium-binding sensor proteins, such as calmodulin (CaM), calcineurin B-like proteins (CBLs), and calcium-dependent protein kinases (CDPKs), which initiate downstream signaling [45]. Furthermore, MAP kinases (e.g., MPK3 and MPK6) can phosphorylate proteins, including transcription factors, in response to stress signals and promote the activation of defense-related genes in the nucleus [14]. Together, these signaling pathways suggest a coordinated interplay among mitochondria, plastids, and the nucleus in orchestrating gene expression essential for cell survival during oxidative stress. Although our study did not directly investigate nuclear gene expression, ANAC017 translocation, or calcium signaling components, these well-established pathways provide a conceptual framework for interpreting the observed stress responses and merit further targeted investigation in future research on the cellular effects of SEO in plants.

Although our study showed that H_2O_2 predominantly accumulates in organelles, this relatively stable molecule is known to not only participate in signaling but also diffuse into the nucleus, where its excess may induce oxidative damage to DNA [46]. While we did not directly assess nuclear H_2O_2 levels, previous findings have demonstrated its ability to permeate cellular membranes—including the nuclear envelope—and to generate DNA lesions. These include oxidative base modifications such as guanine oxidation to 8-oxoG, single- and double-strand breaks, and the subsequent activation of the ATM/ATR–SOG1 signaling cascade, which regulates DNA repair and can induce cell cycle arrest at the G1/S checkpoint or during replication [17]. Alternatively, like other DNA-damaging factors, H_2O_2 could shift the cell cycle toward endoreplication when genome integrity is compromised [47–49]. However, our cytophotometric analysis of cell nuclei from all tested species, following 24 h seedling incubation with IC_{50} SEO concentrations did not reveal any nuclei with DNA content exceeding 4C. This suggests that the level or duration of stress was insufficient to trigger endocycle entry, or alternatively, that meristematic cells of these species do not typically engage in endoreplication under this type of oxidative challenge. Instead, we observed an increase in the population of nuclei in the G1 phase, pointing to the possible activation of the G1/S checkpoint. In plants, this control mechanism, referred to as Principal Control Point 1 (PCP1), integrates signals related to cellular metabolism, energy status (including ATP availability), nucleotide pools, and genomic integrity to determine whether replication should proceed [29,37,38].

Despite the substantial accumulation of reactive oxygen species (ROS) in mitochondria of meristematic root cells treated with SEO—potentially contributing to ATP depletion due to intensified mitochondrial electron transport activity [50]—the energy reserves were likely not sufficiently exhausted to fully activate PCP1. This conclusion is supported by the observation that, although reduced compared to control meristems, a significant proportion of cells still initiated DNA synthesis: approximately 70% of the control level in Fabaceae and 80% in Brassicaceae. Under conditions of severe energy or nutrient limitation, the cell cycle is typically arrested at PCP1, preventing EdU incorporation into nuclear DNA [51]. However, cells exposed to SEO exhibited notably weaker nuclear fluorescence signals from incorporated EdU compared to the control. This reduction may, in part, stem from limited EdU uptake. Prior to incorporation into DNA, EdU must be transported across the plasma membrane via nucleoside transporters and phosphorylated by nucleoside kinases [52]. Our earlier findings of increased lipid peroxidation under SEO treatment suggest impaired membrane integrity, potentially affecting nucleoside transport. Moreover, severe mitochondrial stress could hinder ATP production, further limiting EdU activation via phosphorylation. Nevertheless, since PCP1 did not block entry into S phase, the EdU signal likely reflects reduced replication fork progression and/or a limited number of simultaneously active replicons. This interpretation aligns with previous studies showing that volatile monoterpenes can slow down replication in the meristematic cells of *Brassica campestris* [53]. The observed effects may be linked to oxidative stress signals from mitochondria or plastids, possibly indicating damage to organellar DNA during interphase replication. Such disturbances could indirectly influence the rate of nuclear DNA synthesis [42,53]. Additionally, they may point to replication-associated obstacles within the nucleus itself that require time to resolve. This hypothesis is supported by the strong nuclear labeling of the modified histone variant H2AX (γ -H2AX), which marks sites of double-strand breaks (DSBs). The presence of γ -H2AX confirms that replication slowdown may result from S-phase checkpoints activation in response to DNA damage, most likely mediated by ATM/ATR kinases and the plant-specific transcription factor SOG1—a functional analog of p53 in animal cells—initiating the DNA repair response.

Although γ -H2AX accumulation might also suggest early stages of programmed cell death, it more likely reflects the mild genotoxic potential of SEO, which does not lead to extensive cellular degradation. Hallmarks of advanced cell death, such as the formation of apoptotic-like bodies or DNA laddering observed during electrophoretic DNA separation, were not detected, supporting this interpretation. These findings are consistent with results obtained for meristematic cells of *Lactuca sativa* L. treated with *Backhousia citriodora* L. essential oil, where DNA fragmentation was minimal, despite detectable lesions in the TUNEL assay and the absence of DNA laddering in electrophoretic analysis [54]. In contrast, seedlings of *Lactuca sativa* L. treated with juglone clear DNA showed degradation in the TUNEL assay, accompanied by a characteristic smear on DNA electrophoresis, indicative of extensive damage [55].

Despite the presence of DNA lesions, replication proceeds in SEO-treated cells, with nuclei in all analyzed species progressing to the final stage of DNA synthesis, during which heterochromatin, organized into the chromocenters, is replicated. Interestingly, the proportion of nuclei at this late replication stage (distinguished by clustered fluorescent signals) increased in SEO-treated meristems, which may point to difficulties in repairing DNA within highly condensed chromatin regions [56]. This suggests that the checkpoint system may retain cells at this stage, providing additional time for repair processes to be completed [12]. Nevertheless, further investigation is required to determine whether the accumulation of nuclei at this phase indeed results from checkpoint-mediated de-

lays in response to unresolved replication-associated DNA damage within condensed chromatin regions.

The genotoxic effects observed in this study are likely attributable to the presence of asarones, the principal constituents of SEO [57]. Although no similar studies have examined the impact of *A. calamus* essential oil or its individual components in plant cells, their effects have been assessed in human liver carcinoma (HepG2) cells. In that study, HepG2 cells were incubated with EC₅₀ concentrations of α - and β -asarone, as well as with asarone epoxides—metabolic derivatives of asarones. The results showed that α -asarone, and especially asarone epoxide, exerted stronger biological activity than β -asarone, which is already considered a potent genotoxic agent [58]. DNA strand breaks were detected as early as 1 h after incubation, accompanied by activation of DNA repair mechanisms, as indicated by increased ATM kinase expression 2 h after exposure to asarone epoxide. The findings suggest that the genotoxicity of asarones may stem from their metabolic conversion into reactive epoxides [58] and raise the question of whether similar metabolic activation mechanisms exist in plant cells.

Taken together, our results suggest that while treatment with SEO at IC₅₀ concentration for 24 h induces a measurable genotoxic stress response, the damage remains within a threshold that permits the continuation of replication, albeit with delays and activation of S-phase checkpoints. However, preliminary experiments conducted during IC₅₀ selection revealed that doubling the concentration to $2 \times \text{IC}_{50}$ resulted in more severe nuclear alterations, including chromatin disorganization and impaired replication dynamics. These observations imply that prolonged exposure or higher doses may shift the cellular response toward irreversible damage. Further studies are planned to confirm this dose-dependent effect and to characterize the extent and nature of replication disturbances and genotoxic lesions.

4. Materials and Methods

4.1. Plant Material

Seeds of 4 plant species: faba bean (*Vicia faba* L. subsp. *minor*, cv. Bobas; Danko, HR Sp. z o. o. Choryń, Poland), yellow lupine (*Lupinus luteus* L., cv. Baryt; PHR Sp. z o. o. Tulce, Poland), rapeseed (*Brassica napus* L. cv. Markus; HR Strzelce Sp. z o. o. Strzelce, Poland), and thale cress (*Arabidopsis thaliana* Col-0 (L.) Heynh.; NASC, Nottingham, UK) were germinated on filter paper moistened with deionized water in Petri dishes for 2–3 days at 23 °C in darkness, except *A. thaliana*, which germinated in long-day conditions (16/8 h) due to etiolation sensitivity. Seedlings with equal-sized primary roots (about 2 cm *V. faba*, *L. luteus*, or 1 cm *B. napus* and 0.5 cm *A. thaliana*) were selected for further experiments. In addition to their agricultural importance, species selection was guided by technical criteria relevant to cytological and molecular studies. Among the Fabaceae, *Vicia faba* var. *minor* was chosen for its large genome size, clearly visible interphase nuclei with a reticulate structure and prominent chromocenters, as well as large, easily distinguishable chromosomes suitable for damage assessment. In contrast, *Lupinus luteus* has considerably lower DNA content and displays nuclei with a more dispersed chromatin structure, facilitating the detection of interphase-level damage. Within the Brassicaceae, *Brassica napus*, also characterized by low DNA content, displays a similar chromatin organization and nuclear type, and performs well in immunocytochemical analyses. *Arabidopsis thaliana* was included as a reference model species with a well-characterized genome, enabling future molecular analyses, including gene expression studies.

4.2. Essential Oil Treatment

For performing the treatment, sweet flag essential oil (SEO) (Etja, Elblag, Poland) was used. To decrease the tension between the oil and water phases, the SEO in an emulsified form was prepared. The emulsifier (consisted of C14-18 and unsaturated C16-18—mono- and di-ethoxylated glycerides and ethoxylated *B. napus* oil) was mixed with SEO in a 1:4 (*v/v*) ratio and vigorously mixed by vortexing for 15 min. For seedling incubation, the emulsified SEO was diluted in water to concentrations corresponding to the IC₅₀ values. To ensure a comparable stress level and standardize plant responses, IC₅₀ values were determined individually for each tested species: 0.03% for *V. faba*, 0.025% for *L. luteus*, 0.01% for *B. napus*, and 0.005% for *A. thaliana* [20]. These concentrations represent the doses that inhibited embryonic root growth by 50%. This experimental setup enables a detailed investigation of SEO's mode of action, allowing the identification of physiological and molecular responses under moderate stress conditions. Such conditions do not cause organismal death but instead trigger defense mechanisms and induce metabolic changes. Additionally, this approach facilitates comparisons of cellular behavior across species, helping to determine whether the observed responses are conserved, species-specific, or show patterns characteristic of particular plant families [22]. Seedlings of all four species were incubated on filter paper in tightly sealed with Parafilm Petri dishes, in darkness, at 23 °C for 24 h, using SEO concentrations corresponding to the IC₅₀ values. Seedlings incubated in water and dissolved emulsifier in the concentration used for SEO emulsification were considered as control. Ten root meristems were examined per species (Control series, emulsifier series—E, and SEO series—O), and four experimental repeats were performed, resulting in a total of *n* = 40 root meristems per species per treatment.

4.3. DNA Staining for Cytophotometry

Excised 1 cm long root segments were fixed in Carnoy's solution (99.8% ethanol: glacial acetic acid, 3:1 *v/v*) for 1 h at room temperature, rinsed in absolute ethanol (3 times), and stored in 70% ethanol. Before DNA staining, root tips were rehydrated, hydrolyzed in 4M HCl (for 2h *V. faba*; 1h *L. luteus*, *B. napus*; and 30 min *A. thaliana*) and stained with Schiff's reagent (pararosaniline; Sigma-Aldrich, St. Louis, MI, USA) for 1 h using the standard Feulgen method [59]. Root meristems were cut off, squashed in a drop of 45% acetic acid onto microscopic slides (Menzel-Gläser, Thermo Fisher Scientific, Waltham, MA, USA), and placed on the surface of a dry ice cube. Following freezing with dry ice, coverslips were removed, and the preparations were rinsed in 70% ethanol, air-dried, and covered in a drop of Canada balsam.

4.4. Detection of a Replication Process

The Click-iT[®] EdU Alexa Fluor[®] 555 Imaging Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to visualize DNA synthesis. Seedlings were incubated on a watch glass in 10 µM 5-ethynyl-2'-deoxyuridine (EdU) solution for 30 min, at room temperature in darkness. Then, roots were fixed in 0.01 M PBS-buffered 4% paraformaldehyde solution (4 °C; pH 7.4) for 40 min and rinsed twice in 0.01 M PBS buffer. Next, root tips were macerated with a 0.1 M citrate-buffered mixture of 2.5% pectinase, 2.5% pectolyase, and 2.5% cellulase (Sigma Aldrich) at pH 5.0 and 37 °C for 30 min (*V. faba* and *L. luteus*), 20 min (*B. napus*), or 15 min (*A. thaliana*). Rinsed twice in PBS, meristems were squashed in a drop of 0.01 M PBS onto Super Frost Plus microscope slides (Menzel-Gläser). Following freezing with dry ice, coverslips were removed, and slides were air-dried. After washing with 0.01 M PBS, DNA replication in nuclei was visualized by the Click-iT reaction carried out according to the manufacturer's protocol. After the reaction, slides were washed with 0.01 M PBS, stained with 15 µM 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for

15 min, rewashed with 0.01 M PBS, and mounted in 0.01 M PBS/glycerol/DABCO (2.3% diazabicyclo [2.2.2]octane) mixture.

4.5. Detection of Apoptosis-like Programmed Cell Death (AI-PCD)

Due to the comparable replication patterns observed across all four species and technical limitations of immunocytochemical analyses, two representative species—one from Fabaceae and one from Brassicaceae—were selected for further investigation. Root meristems of *V. faba* and *B. napus* were fixed in 0.01 M PBS-buffered 4% paraformaldehyde for 40 min at 4 °C, rinsed twice in 0.01 M PBS buffer, and macerated with a 0.1 M citrate-buffered enzyme mixture of 2.5% pectinase, 2.5% pectolyase, and 2.5% cellulase (Sigma Aldrich) at 37 °C for 30 min (*V. faba*) or 20 min (*B. napus*). A TUNEL assay was performed according to the manufacturer's instruction (Click-iT® TUNEL Alexa Fluor® 488 Imaging Assay (Thermo Fisher Scientific). Cell nuclei were stained with DAPI (15 µM 4',6-diamidino-2-phenylindole; Sigma-Aldrich) for 15 min and rinsed again with 0.01 M PBS (3 times for 10 min each). Microscopic slides were mounted in 0.01 M PBS/glycerol/DABCO mixture.

DNA was isolated from root tips using NucleoSpin Plant II, Mini kit for DNA from plants (Macherey-Nagel) according to the manufacturer's instructions. DNA samples (2 µg DNA each) were separated in agarose gel electrophoresis (1%) in a 1X Tris-acetate-EDTA (TAE) buffer containing ethidium bromide (1 mg · mL⁻¹) at 100 V for 3 h at RT. Gel was visualized using ProXima 2750 imaging platform (Isogen Life Sciences, De Meern, The Netherlands).

4.6. Immunocytochemical Staining of γ -Phosphorylated H2AX Histones

The same two representative species were used for this part of the study, as justified in the previous section. Apical parts of *V. faba* and *B. napus* roots, fixed in 0.01 M PBS-buffered 4% paraformaldehyde (4 °C, 40 min) and macerated with a 0.1 M citrate-buffered enzyme mixture of 2.5% pectinase, 2.5% pectolyase, and 2.5% cellulase (Sigma Aldrich) at 37 °C for 30 min (*V. faba*) or 20 min (*B. napus*), were squashed onto Super Frost Plus microscope slides (Menzel-Gläser) and frozen on a dry ice cube (as described above in the procedure for identifying the replication process). Then air-dried samples were pretreated with 0.01 M PBS-buffered 5% bovine serum albumin (BSA, Sigma Aldrich) and 4% Triton X-100 (Sigma Aldrich) at room temperature for 50 min, and subsequently incubated overnight at 4 °C in darkness and a humidified atmosphere with primary rabbit polyclonal antibodies against human γ -H2AX histones phosphorylated at Ser139 (Thermo Fisher Scientific), diluted 1:750 in antibody buffer (1% BSA; 0.3% Triton X-100 in 0.01 M PBS). In the next day, slides were rinsed with 0.01 M PBS (3 times for 10 min) and incubated at 23 °C in darkness for 90 min with the secondary mouse, anti-rabbit antibodies, conjugated with Alexa Fluor® 488 dissolved in 1:600 ratio in antibody buffer. After rinsing with 0.01 M PBS (3 times for 10 min each), cell nuclei were stained with DAPI (15 µM 4',6-diamidino-2-phenylindole; Sigma-Aldrich) for 15 min and rinsed again with PBS (3 times for 10 min each). After that, the slides were embedded in 0.01 M PBS/glycerol mixture (9:1) with 2.3% DABCO.

4.7. H₂O₂ and O₂^{•-} Detection in Root Meristematic Cells

For H₂O₂ detection, seedlings of *V. faba*, *L. luteus*, *B. napus*, and *A. thaliana* after 24 h incubation in water, emulsifier, and IC₅₀ emulsified SEO were incubated for 1 h at room temperature in 1 mg · mL⁻¹ diaminobenzidine tetrachloride solution (DAB-HCl; Sigma-Aldrich) in 0.01 M PBS buffer and rinsed twice in 0.01 M PBS buffer. Then, excised root tips were fixed in 4% paraformaldehyde buffered with 0.01 M PBS for 40 min at 4 °C, rinsed twice in 0.01 M PBS, and macerated with a 0.1 M citrate-buffered enzyme mixture (2.5% pectinase, 2.5% pectolyase and 2.5% cellulase; Sigma Aldrich) at pH 5.0 and 37 °C for

30 min (*V. faba* and *L. luteus*), 20 min (*B. napus*), or 15 min (*A. thaliana*). The samples were then washed and embedded in 0.01 M PBS/glycerol mixture (9:1) with 2.3% DABCO.

To visualize $O_2^{\bullet-}$ seedlings of *V. faba* and *B. napus* after 24 h incubation in water, emulsifier, and IC_{50} emulsified SEO were incubated for 10 min at room temperature in a 3 mM 0.01 M PBS-buffered nitroblue tetrazolium (NBT; Sigma Aldrich) and rinsed twice in 0.01 M PBS buffer after the incubation stopped. The next steps considering slide preparation were similar to those for DAB staining.

4.8. Microscopic Observations, Measurements, and Analyses

DNA content was evaluated with a Jenamed 2 microscope (Carl Zeiss, Jena, Germany). The extinction of Feulgen-stained cell nuclei was measured at 565 nm and calibrated in arbitrary units, taking the values recorded for half-telophases and prophases from control roots as reference standards of 2C and 4C DNA levels, respectively. A computer program (Forel, Łódź, Poland) was used for image analysis using micro densitometry to evaluate DNA content from 5000 nuclei from five meristems.

Z-stacks of EdU-treated cells were acquired using a 0.3 μ m step size under oil immersion using Leica Laser Scanning Confocal Microscopy (LSCM) SP8 platform (Leica Microsystems, Wetzlar, Germany) using 100 $\times$ /1.40 OIL lens, with the use of Laser Line Supercontinuum Visible (555 nm) and UV (405 nm) Diode laser and visualized using Leica LAS X software ver. 2.0.2.15022. Proportions [%] of early-, mid-, and late-S-phase cells in meristems of *V. faba* seedlings, calculated by combining microfluorimetric quantitation of DNA contents in DAPI-stained cell nuclei and visual EdU fluorescence analysis.

Observations and analyses of DAB, NBT, TUNEL and anti- γ -phosphorylated H2AX histone visualized cells were made using Nikon Eclipse E600W fluorescence microscope (Nikon, Tokyo, Japan) equipped with U2 filter (UVB light; λ = 340–380 nm) for DAPI, B2 filter (blue light; λ = 465–496 nm) for Alexa Fluor[®] 488, and G2 filter (green light; λ = 540/25 nm) for Alexa Fluor[®] 555. All images were recorded at the same time of integration using a DS-Fil CCD camera (Nikon). Quantitative color or fluorescence intensity analyses were made in ImageJ software ver. 1.54p [60] after converting color images into greyscale and expressed in arbitrary units.

Analysis of DNA gel electrophoresis was performed using GelAnalyzer 23.1.1 (available at www.gelanalyzer.com) by Istvan Lazar Jr., PhD and Istvan Lazar Sr., PhD. After loading the gel image, lanes and bands were automatically detected by the program. The smear volumes were measured by adjusting the band visible on the lane profile, namely, the threshold covering the band visible as a peak on the lane plot has been moved below the line and stopped at the nearest plateau. The smear volume was expressed in the Analysis info window in the Raw volume column.

4.9. Statistical Analyses

Statistical analyses and outcomes visualization were performed in GraphPad Prism 10 software. Differences between groups were evaluated using Student's *t*-test or Mann–Whitney test. A *p*-value ≤ 0.05 was considered statistically significant and marked with asterisks (*) or hash (#).

5. Conclusions

Our study shows that meristematic cells of primary roots from four species—*Vicia faba*, *Lupinus luteus*, *Brassica napus*, and *Arabidopsis thaliana*, representing Fabaceae and Brassicaceae—exhibit a common response to SEO at its IC_{50} concentration. This includes oxidative stress, with ROS primarily accumulating in mitochondria and plastids, but also affecting the nucleus (Figure 15).

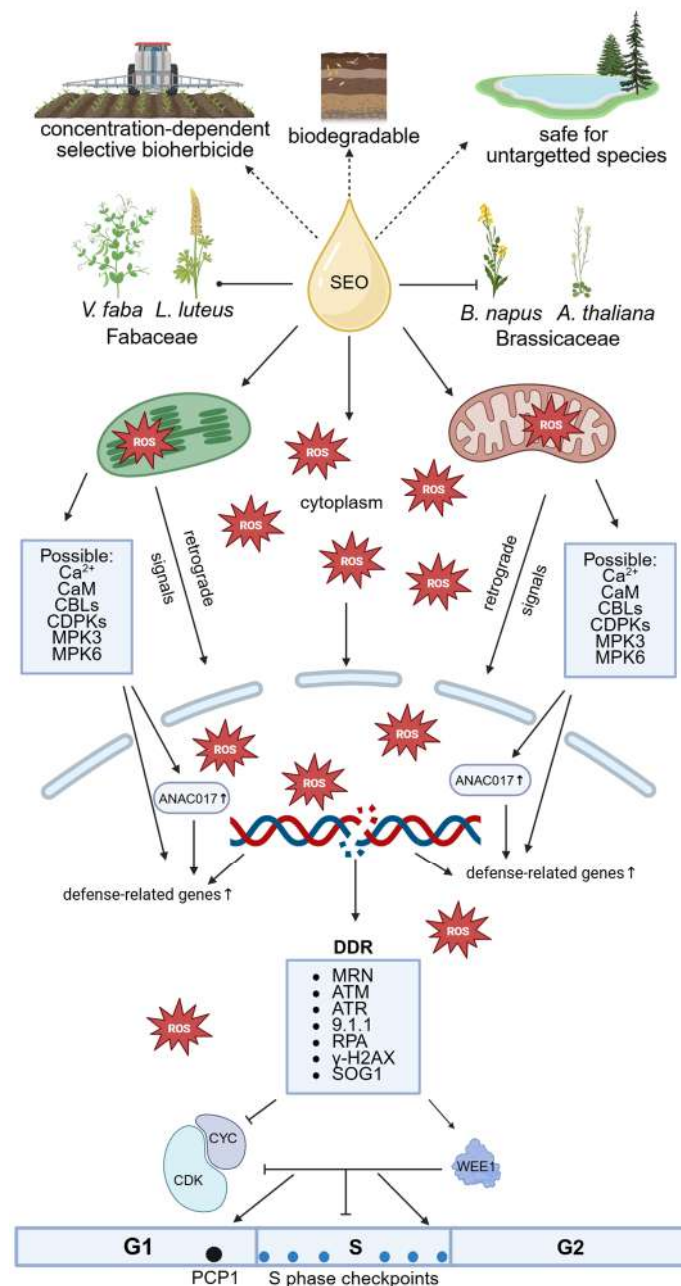


Figure 15. Proposed mechanism of action of *Acorus calamus* essential oil (SEO) at IC₅₀ concentration in root meristematic cells of Fabaceae and Brassicaceae species. SEO induces organelle-specific oxidative stress, leading to DNA damage, activation of cell cycle checkpoints, and altered replication dynamics without triggering extensive cell death. These effects underpin its potential as a selective, biodegradable, and environmentally safe bioherbicide for sustainable weed management. Depending on concentration, SEO exhibits species-specific selectivity, enabling targeted suppression of Brassicaceae weeds in Fabaceae-dominated cropping systems (explanation of abbreviations in the work test). Created with Biorender.com.

Oxidative stress, along with the direct action of SEO components, causes DNA double-strand breaks that trigger checkpoint activation and repair mechanisms, delaying cell cycle progression. As a result, more cells accumulate in G₁ phase, DNA replication slows due to fewer active replicons or slower fork progression, and heterochromatin replication is impaired due to chromatin compaction. Despite evident genotoxicity, the absence of apoptotic-like fragmentation or endoreduplication suggests that SEO does not induce extensive cell death or trigger genome duplication. Instead, oxidative stress disrupts S

phase progression, with species differing in severity, not in response type. Given the importance of accurate DNA replication for mitosis, our ongoing research investigates whether this replication stress impacts mitotic structures or chromosome behavior.

These findings support the further development of *Acorus calamus* essential oil (SEO) as a sustainable weed management tool. Future studies should first apply multi-omics approaches to clarify molecular mechanisms, followed by expanded species screening under laboratory and field conditions, and the optimization of application parameters and formulations.

Taken together, our results support the use of SEO as effective, selective, biodegradable, and environmentally responsible weed control tools, enabling targeted suppression of weeds or invasive plants while sparing crops and helping to address herbicide resistance. Such approaches hold promise for regulatory approval and societal acceptance, while potentially reducing chemical inputs, lowering production costs, and enhancing the long-term sustainability and resilience of agroecosystems.

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Abbreviations

The following abbreviations are used in this manuscript:

9-1-1 Complex	RAD9-RAD1-HUS1 Complex
ATM	Ataxia-Telangiectasia Mutated Kinase
ATR	Ataxia-Telangiectasia and Rad3-Related Kinase
BSA	Bovine Serum Albumin
Ca ²⁺	Calcium ions
CaM	Calmodulin
CBL	Calcineurin B-Like Protein
CDK	Cyclin-Dependent Kinase
CDPK	Calcium-Dependent Protein Kinase
CKI	Cyclin-Dependent Kinase Inhibitor
Cyc	Cyclin
DAB	3,3'-Diaminobenzidine
DABCO	2,3% diazabicyclo [2.2.2]octane;
DAPI	4',6-Diamidino-2-Phenylindole
DDR	DNA Damage Response
EdU	5-Ethynyl-2'-Deoxyuridine
H ₂ O ₂	Hydrogen Peroxide

IC ₅₀	Half-inhibitory Concentration
KRP	Kip-Related Protein
MPK	Mitogen-Activated Protein Kinase
MRN	MRE11-RAD50-NBS1 Complex
NBT	Nitro blue tetrazolium chloride
O ₂ ^{•−}	Superoxide Anion Radical
PBS	Phosphate-Buffered Saline
AL-PCD	Apoptosis-Like Programmed Cell Death
PCP1	Principal Control Point 1
Rb	Retinoblastoma Protein
ROS	Reactive Oxygen Species
RPA	Replication Protein A
SEO	<i>Acorus calamus</i> essential oil
SOG1	Suppressor of Gamma Response 1
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling
γ-H2AX	Phosphorylated Histone H2AX

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Article

Mitotic Disruption and Cytoskeletal Alterations Induced by *Acorus calamus* Essential Oil: Implications for Bioherbicidal Potential

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Abstract

Essential oils are increasingly recognized as promising agents for sustainable weed control due to their selectivity and complex modes of action. This study evaluated the effects of *Acorus calamus* essential oil (SEO) on mitosis in two Fabaceae species (*Vicia faba*, *Lupinus luteus*) and two Brassicaceae species (*Brassica napus*, *Arabidopsis thaliana*) treated with species-specific IC₅₀ concentrations (0.03%, 0.025%, 0.01%, and 0.005%, respectively). Previous research showed that SEO induces oxidative stress and S-phase delay via genome instability. Here, SEO consistently disrupted mitosis across all species, reducing mitotic index by 50–60%, decreasing Cdc2 (CDKA homolog) levels, and causing chromosomal aberrations, including uneven chromatin condensation, sticky chromosomes, bridges, and micronuclei. Cells accumulated in metaphase and exhibited abnormal karyokinetic and cytokinetic spindles. Immunolabeling revealed thick, tightly packed microtubules and actin filaments, indicating excessive stabilization and impaired reorganization. Epigenetic regulation was also affected: H3T3 phosphorylation was abnormally strong, widely distributed, and persistent into anaphase/telophase, while H3S10Ph intensity was weakened. These results suggest that SEO targets multiple components of mitotic machinery and epigenetic control, regardless of species. The observed selectivity depends on dosage, not mechanism. This multi-targeted action may limit the development of plant resistance, supporting the potential of SEO as a bioherbicide in sustainable agriculture.

Keywords: phytotoxicity; mitotic index; chromosomal aberrations; CDKA; epigenetics; cytoskeleton



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1. Introduction

The extensive use of chemical pesticides, driven by global demand for high crop yields and aesthetic products, has significant detrimental effects on the environment and human health. Due to their poor biodegradability, crops and soil are abundant in their toxic remains. Nowadays, increasing efforts are being undertaken to reduce the use of chemical pesticides, which induce resistance in target organisms and hamper the well-being of non-targeted ones [1–3]. Unfortunately, the sophistication of the development and testing process of new, safer substances and the long legislation procedures in light of systematic pesticide withdrawal from the market cast a shadow on efficient crop production. The ambitious idea of replacing or even reducing the use of synthetic pesticides with biologically originated

compounds in a short time is a global challenge [4]. Despite the serious problems, such as the intensification of pest metabolism due to global warming, weed infestation causes enormous crop losses exceeding twice those caused by pests [5]. Weeds compete with crop plants for shared resources such as light, water, and nutrients, which depletion is detrimental for crops, especially during the initial growth stages. Recent studies indicate that weeds are responsible for up to 50% of crop losses even when the best practices of weed management are applied [6]. Moreover, in slow-growing legumes, crop losses are said to be up to even 97% [7].

Some crop plants can control the weed competitors by excreting chemical substances that affect the growth of the neighboring plants. The phenomenon called allelopathy is based on a chemical interaction between two plant species where one excretes allelochemicals represented by plant secondary metabolites, which are detrimental to the second one. Allelopathy has been observed for many plant species [8–13]. In recent years, increasing attention has been paid to allelochemicals of the benzoxazinoid family produced by rye, wheat, and maize [14]. Allelopathy is then a promising strategy in modern, environmentally friendly crop protection from weeds.

Essential oils (EOs) are other allelochemicals originating from plant secondary metabolism. These volatile substances exhibiting allelopathic activities are complex mixtures of 20–60 active ingredients classified as terpenes, phenylpropanoids, alcohols, aldehydes, and esters [15,16]. They have attracted considerable attention due to their diverse bioactive properties [8,17–22]. Beyond general phytotoxic effects, these metabolite blends can also interfere with fundamental cellular functions [10,11,21,23–27]. At the organismal level, EOs commonly inhibit seed germination, root and shoot elongation, and leaf development, as shown, inter alia, for *Citrus aurantiifolia* [28] and *Mentha longifolia* EO [29] and for many other EOs widely described by Verdeguer et al. [16]. These morphological effects result from disruptions of fundamental physiological processes, including photosynthesis, mitochondrial respiration (e.g., by *Origanum vulgare* EO [23] and cinnamaldehyde in cinnamon oil [24]), and hormonal regulation (e.g., by *Artemisia argyi* extract [30]). At the cellular level, EOs induce oxidative stress through excessive production of reactive oxygen species (ROS), lipid peroxidation, and destabilization of membranes and ion balance, as reported for lemongrass, cinnamon, peppermint, well as for individual compounds such as carvacrol and eugenol. Terpene-rich EOs also impair plant growth by inhibiting mitosis, leading to reduced DNA synthesis, a lower mitotic index, phase-specific arrest, and chromosomal aberrations caused by cytoskeletal dysfunctions [10,16,17,20,23,25,31,32]. For instance, *Schinus terebinthifolius* and *Schinus molle* EOs interfere with the cell cycle by disrupting chromatin condensation and spindle organization during mitosis [33]. Taken together, these multi-level phytotoxic effects highlight the potential of EOs as promising candidates for environmentally safe bioherbicide formulations in sustainable agriculture.

An essential oil possessing intriguing properties is produced in rhizomes of sweet flag (*Acorus calamus* L.) [34–36]. It consists of different classes of chemical substances, where phenylpropanoids, including α -, β -, and γ -asarones, are the most abundant. Numerous studies have confirmed the medicinal properties of the sweet flag essential oil (SEO), which has a long history of use in traditional Chinese medicine [35–38]. However, asarones, which are abundantly present in the oil, can exhibit either pro- or antioxidative activity depending on the concentration [39]. This dualistic mode of action has become a subject of scientific debate, particularly in light of its potential genotoxic and cytotoxic effects, which remain inconclusive due to insufficient data [40]. Studies on the impact of SEO on plant growth remain limited. However, available data indicate its selective inhibitory effects on seed germination and early seedling development, highlighting it as one of the most potent EOs in this context [12,22]. At the cellular level, SEO at its IC₅₀ concentration

(causing a 50% reduction in root growth), provokes oxidative stress characterized by excessive accumulation of ROS, enhanced lipid peroxidation, and the induction of DNA double-strand breaks. This leads to replication stress, manifested by reduced proportion of cells entering S phase, and impaired progression through DNA synthesis, primarily due to diminished replication dynamics within condensed heterochromatin. Notably, despite these genotoxic effects, SEO does not trigger endoreduplication, apoptotic DNA fragmentation, or extensive cell death [22,41]. Notably, SEO influence on cell cycle regulation in meristematic tissues is of particular interest, as these regions play a critical role in plant growth and development [42,43]. Within the cell cycle, multiple potential targets may be affected by EO constituents, including molecular regulators of cell cycle progression, the integrity of the dynamic cytoskeleton, and structural remodeling of chromatin [16]. Gaining insight into the mechanisms by which substances contained in SEO affect cell proliferation is essential for evaluating both their biological relevance and potential applications in crop management and plant protection.

Plant cell proliferation is highly sensitive to environmental factors and relies on conserved eukaryotic mechanisms with plant-specific features, regulated mainly at the G1/S and G2/M transitions [44–49]. Progression through the cycle depends on cyclin-dependent kinase/cyclin complexes (CDK/Cyc) [50–55], whose activity is modulated by CDK-activating kinases (CAKs), CDK/Cyc inhibitors (CKIs) [53,56–63], antagonistic regulators such as WEE1 kinase and CDC25-like phosphatase [64–66], and other protein phosphatases (PP1/PP2A). The RBR/E2F/DP pathway also plays a central role, linking cell cycle control with transcription of S-phase genes [67–69].

In early G1, CDKA/CycD complexes, once activated by CAK and released from CKI or WEE1 inhibition, phosphorylate RBR proteins, thereby releasing E2F/DP to induce transcription required for DNA replication. Replication origin licensing and checkpoints mediated by ATM/ATR kinases and CHK-like proteins then secure accurate initiation and fidelity of S phase [52,67,70–73].

During G2, increasing levels of CycA, B, and D form complexes with CDKA (the plant homolog of yeast and animal Cdc2/CDK1) or with the plant-specific CDKB to promote mitotic entry [45,66–69]. These complexes are suppressed by CKIs or by WEE1-mediated phosphorylation and activated by CAK [74–76]. A CDC25-like phosphatase may also remove inhibitory phosphates, although in higher plants only a truncated homolog has been identified, retaining the catalytic but lacking the regulatory domain, suggesting a plant-specific mechanism of G2/M regulation. CDC25 activity itself is antagonized by PP1 and PP2A [69,77].

Active CDK/Cyc complexes further phosphorylate CKIs, preventing secondary repression, and act on substrates that drive mitosis. These include transcription factors such as MYB3R, which activate genes encoding mitotic proteins (e.g., CDC20, CycA, CycB, NACK1) [54,78], as well as structural components: lamins (nuclear envelope breakdown), histone H1 (chromosome condensation), and nucleolin (nucleolar disorganization) [79–81]. These molecular reactions control the distribution of the replicated DNA between daughter cells, with the participation of dynamic cytoskeletal rearrangements [82,83].

In plant cells, early mitotic events are coordinated by the preprophase band (PPB), a transient microtubule–actin structure that marks the future division plane [83]. Although it disassembles at prophase, its positional information is retained through cortical landmarks and cytoskeletal reorganization, ensuring correct spindle orientation and division plane placement. During prophase, microtubules around the nuclear envelope reorganize into a bipolar spindle, which in plants forms without centrosomes; instead, microtubule-rich regions at opposite nuclear sides act as poles interconnected by microtubule-associated proteins (MAPs) and motor proteins [83]. Actin filaments form a supportive cage around

the spindle, maintaining its orientation and stability [84–86]. After nuclear envelope breakdown, microtubules rapidly polymerize and attach to kinetochores, forming stable kinetochore fibers reinforced by γ -tubulin ring complex (γ -TuRC). In metaphase, augmin-dependent γ -TuRC recruitment drives branching nucleation, enhancing spindle robustness and chromosome alignment [83]. During anaphase, chromatids separate through coordinated microtubule depolymerization at both plus and minus ends, while central spindle microtubules are continuously regenerated and stabilized. Kinesin-14 motors generate sliding forces that maintain spindle integrity and elongation [83]. Finally, in telophase, cytokinesis is initiated by phragmoplast formation, a dynamic structure of antiparallel microtubules, actin filaments, and associated proteins that direct vesicles with cell wall precursors to the division site, facilitating cell plate assembly and the formation of the new cell wall. MAP65 proteins cross-link antiparallel microtubules, maintaining phragmoplast integrity and guiding its expansion until the cell plate fuses with the parental plasma membrane [83,86].

Alongside cytoskeletal reorganization, chromatin undergoes major structural changes during mitosis. In the G2/M transition, previously decondensed and transcriptionally active chromatin compacts into mitotic chromosomes through the action of structural maintenance of chromosome (SMC) complexes: condensins drive condensation, while cohesins hold sister chromatids together until anaphase. Together, they provide the scaffold required for chromosome architecture and accurate segregation [87]. An early epigenetic event in mitotic chromatin is histone H3 phosphorylation at threonine 3 (H3T3Ph) by Haspin kinases. In plants, H3T3Ph is broadly distributed and promotes chromatin condensation and CPC (chromosome passenger complex) recruitment. CPC, which accumulates at kinetochores before nuclear envelope breakdown, ensures centromeric localization of Aurora B kinase, a key mitotic regulator required mainly for division initiation [84,88,89]. Once recruited, Aurora B phosphorylates histone H3 at serine 10 (H3S10Ph), a conserved modification appearing in late G2 within pericentromeric heterochromatin and disappearing in telophase [89,90]. H3S10Ph supports condensation and facilitates cohesin release from chromosome arms, promoting sister chromatid resolution. Proper centromeric positioning of Aurora B stabilizes kinetochore–microtubule attachments, which is essential for accurate chromosome segregation [88,89].

As in other eukaryotes, plant mitosis is safeguarded by the spindle assembly checkpoint (SAC), which delays anaphase until all kinetochores are properly attached to spindle microtubules, thereby ensuring accurate chromosome segregation and genome stability [83]. Erroneous attachments activate the SAC and trigger formation of the mitotic checkpoint complex (MCC), consisting of MAD2, BMF3, and CDC20. In this complex, CDC20 is sequestered and unable to activate the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase required for separase activation and chromatid separation. SAC also cooperates with the chromosome passenger complex (CPC) to correct improper kinetochore–microtubule interactions, ensuring that APC/C activation occurs only when all chromosomes are correctly aligned under bipolar tension [88]. This multilayered regulation highlights the essential role of SAC in error-free mitotic progression.

This study aimed to elucidate the bioherbicidal potential of SEO by examining its effects on mitosis, the final stage of the plant cell cycle. Despite documented bioactivity in animal models and *in vitro* systems, the cellular and molecular mechanisms of SEO in plants remain poorly understood, highlighting the novelty of this research [39,42,91]. Using a model previously applied to S-phase studies, we investigated four plant species selected to represent both crops and weeds. The Fabaceae crops *Vicia faba* L. var. *minor* and *Lupinus luteus* L., characterized by large seeds and slow early growth, are particularly susceptible to weed competition and limited selective herbicides [92,93]. The Brassicaceae

species *Brassica napus* L. and *Arabidopsis thaliana* (L.) Heynh were chosen as representatives of undesirable plants in crop systems, while *B. napus* volunteer seedlings can threaten legume production. *A. thaliana* serves both as a common weed and a well-established model organism for future molecular studies [94]. Species-specific IC₅₀ values of SEO were determined to allow for comparisons under equivalent stress conditions [22,41].

We hypothesized that SEO disrupts mitosis by altering the abundance of key cell cycle regulator Cdc2 (the conserved homolog of plant CDKA), spindle organization, and mitotic chromatin structure, and that these effects may vary among species, revealing taxon-specific responses or family-level patterns. To test this, we assessed the abundance of Cdc2, mitotic indices, aberrations in mitotic figures, spindle microtubule and actin filament organization, and histone phosphorylation at H3T3 and H3S10. This framework provides both a mechanistic understanding of SEO action in plant cells and insights into its potential as a selective, eco-friendly bioherbicide.

2. Results

2.1. Effects of SEO on Mitotic Activity and Chromosomal Integrity in Root Meristematic Cells

Mitosis represents the shortest phase of the cell cycle, and cell divisions in the root meristem occur asynchronously and with varying intensity among plant species. Consequently, in control root meristems, the mitotic index (MI) was relatively low and species-specific, averaging approximately 17% in *V. faba*, 9% in *L. luteus*, 11% in *B. napus*, and 3% in *A. thaliana* (Figure 1A–D). The predominance of early mitotic stages among dividing cells reflects the inherently extended duration of prophase, which is the longest phase of mitosis. Accordingly, prophase and metaphase accounted for 61% and 15% of mitotic figures in *V. faba*, 44% and 23% in *L. luteus*, 50% and 17% in *B. napus*, and 33% and 29% in *A. thaliana*, respectively (Figure 1(A'–D')). Therefore, the later mitotic stages (anaphase and telophase) collectively constituted a smaller proportion: 24% in *V. faba*, 33% in *L. luteus*, 33% in *B. napus*, and 38% in *A. thaliana* (Figure 1(A'–D')).

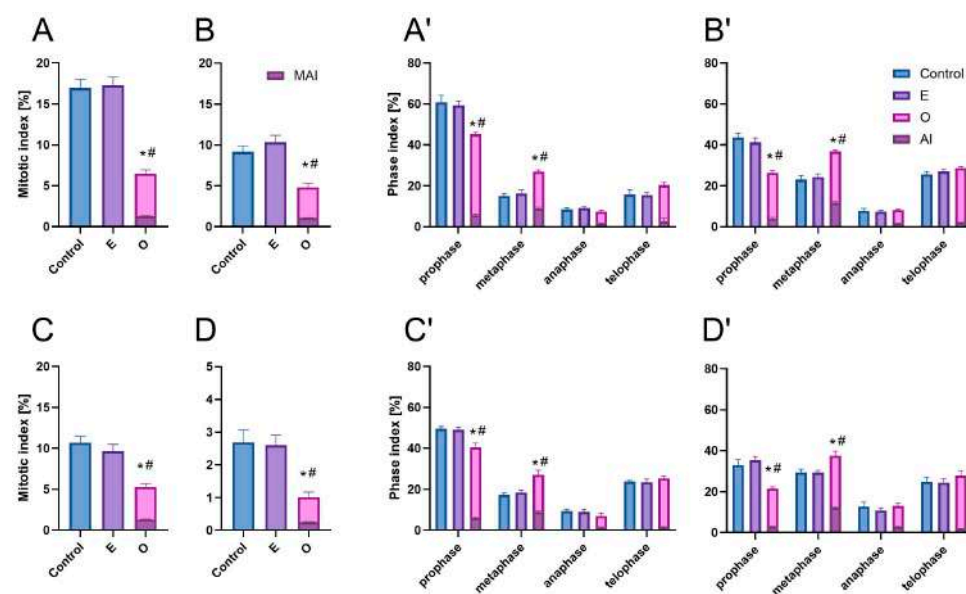


Figure 1. Mitotic indices in root meristem cells of (A) *V. faba*, (B) *L. luteus*, (C) *B. napus*, (D) *A. thaliana*, and phase indices (A'–D', respectively), including mitotic aberration indices—MAI and phase aberration indices—AI, after 24 h seedling incubation in water—Control, emulsifier solution—E, or emulsified SEO at the IC₅₀ concentration—O. Data are presented as mean indices $\pm$ SEM from three biological replicates. Statistical differences were assessed using Student's *t*-test at $p \leq 0.05$. Asterisk (*) and hash (#) marks denote significant differences compared to the control and the emulsifier, respectively.

The emulsifier, used to prepare a uniformly distributed SEO emulsion in the culture medium where the seedlings were grown, had no statistically significant effect on the mitotic index (Figure 1A–D) or on the distribution of mitotic phases (Figure 1(A'–D')). Mitotic figures in both control and emulsifier-treated root meristems displayed proper morphology.

Despite interspecific differences in chromosome number, size, and morphology (compare Figures 2A–D, 3A–D, 4A–D and 5A–D), chromosomes in all four species appeared structurally normal, without signs of breakage, uneven chromatin condensation, or under-replicated chromatid fragments. They aligned correctly on the metaphase plate and segregated faithfully, forming two genetically balanced daughter nuclei, as previously demonstrated in the analysis of interphase nuclei [41].

Following exposure to SEO, notable alterations were observed in the mitotic parameters of the root meristems. The mitotic index significantly declined to an average of 6.5% in *V. faba* (38% of control), 5% in *L. luteus* (just over 50% of control), 5% in *B. napus* (just under 50% of control), and 1% in *A. thaliana* (33% of control) (Figure 1A–D). The distribution of cells across mitotic phases also changed, particularly within early mitosis. All species exhibited a reduction in the proportion of prophase cells (by 15 and 17% in Fabaceae or 9 and 12% in Brassicaceae), accompanied by an increase in metaphase cells (by 12 and 14% in Fabaceae or 10 and 8% in Brassicaceae) (Figure 1(A'–D')).

Moreover, in SEO-treated root meristems of all four species, alongside mitotic figures displaying a normal appearance, cells were also observed to exhibit similar chromosomal structural abnormalities at consecutive stages of mitosis. In prophase, chromatin displayed an uneven degree of condensation, with clearly demarcated, intensely Feulgen-positive regions, likely corresponding to heterochromatic domains (compare figures: Figures 2E,F, 3E,F, 4E,F and 5E,F vs. Figures 2–5A). Metaphase abnormalities were diverse in nature. One such disturbance involved telomere clustering, most clearly observed in *V. faba* due to its large chromosomes (Figure 2G), or misaligned and scattered chromosomes at the metaphase plate, as seen in *B. napus* (Figure 4G). Other anomalies included excessively condensed chromosomes densely packed within the metaphase plate (Figures 2H, 3G, 4H and 5G), or, conversely, chromosomes that appeared insufficiently condensed for this stage or already undergoing premature decondensation despite not having segregated to the poles (Figures 2I,J, 3H,I, 4I and 5H).

In anaphase, chromatid separation appeared impaired, most notably in *V. faba*, where sticky telomeric ends were clearly visible at both early and late stages of anaphase (Figure 2K–N). Similar, though less distinct, abnormalities were also observed in the remaining species, likely due to their smaller chromosomes (Figures 3J, 4J,K and 5I–K). These segregation defects often resulted in the formation of chromosomal bridges or micronuclei during telophase (Figures 2O,P, 3K,L, 4K and 5K), as well as in atypical patterns of chromatin condensation in telophase nuclei (Figures 4L and 5L).

The percentage of mitotic figures exhibiting abnormalities or chromosomal aberrations was similar across all studied species, averaging around 20–25% of dividing cells (Figure 1A–D). The highest proportion of defects (slightly over 30%) was observed during metaphase (Figure 1(A'–D')).

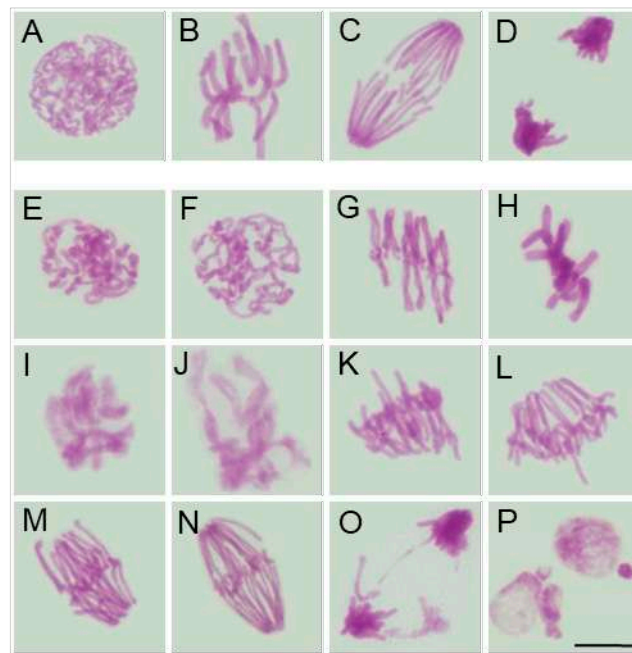


Figure 2. Mitotic figures in root meristem cells of *V. faba* after 24 h seedling incubation in water—Control (prophase: (A); metaphase: (B); anaphase: (C); telophase: (D)) and in emulsified SEO at the IC₅₀ concentration (prophase with uneven chromosome condensation and Feulgen-positive foci: (E,F); metaphase with some telomere clustering: (G); metaphase with excessively condensed chromosomes: (H); metaphase with insufficiently condensed chromosomes: (I,J); early and late anaphase with sticky telomeric ends: (K,L) and (M,N) telophase with chromosomal bridges: (O); post telophase with micronuclei: (P)). Scale bar: 10 μ m.

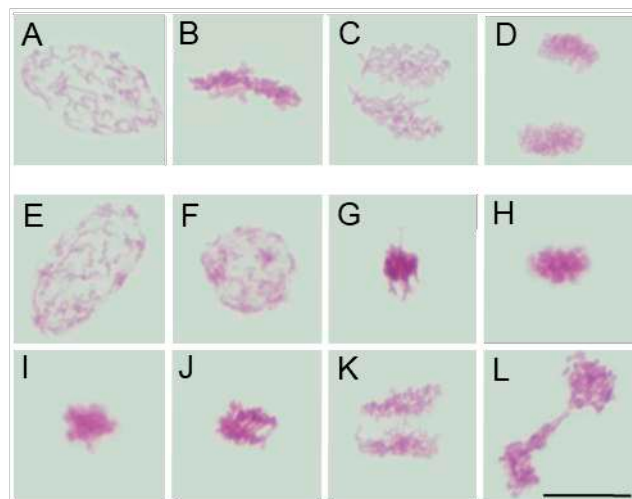


Figure 3. Mitotic figures in root meristem cells of *L. luteus* after 24 h seedling incubation in water—Control (prophase: (A); metaphase: (B); anaphase: (C); telophase: (D)) and in emulsified SEO at the IC₅₀ concentration (prophase with uneven chromosome condensation and Feulgen-positive foci: (E,F); metaphase with excessively condensed chromosomes: (G); metaphase with insufficiently condensed chromosomes: (H); decondensed and condensed anaphase with sticky telomeric ends: (I,J); early and late telophase with chromosomal bridges: (K,L)). Scale bar: 10 μ m.

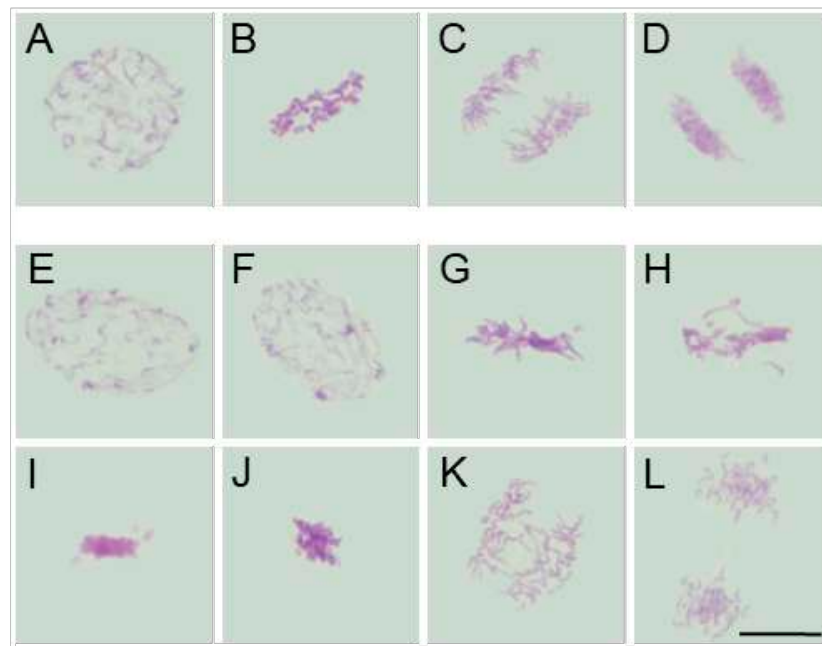


Figure 4. Mitotic figures in root meristem cells of *B. napus* after 24 h seedling incubation in water—Control (prophase: (A); metaphase: (B); anaphase: (C); telophase: (D)) and in emulsified SEO at the IC₅₀ concentration (prophase with uneven chromosome condensation and Feulgen-positive foci: (E,F); metaphase with excessively condensed, misaligned and scattered chromosomes: (G,H); metaphase with insufficiently condensed chromosomes: (I); condensed anaphase with sticky telomeric ends: (J); anaphase with sticky telomeric ends and chromosomal bridges: (K); telophase with a chromosomal bridge and atypical chromatin condensation: (L)). Scale bar: 5 μ m.

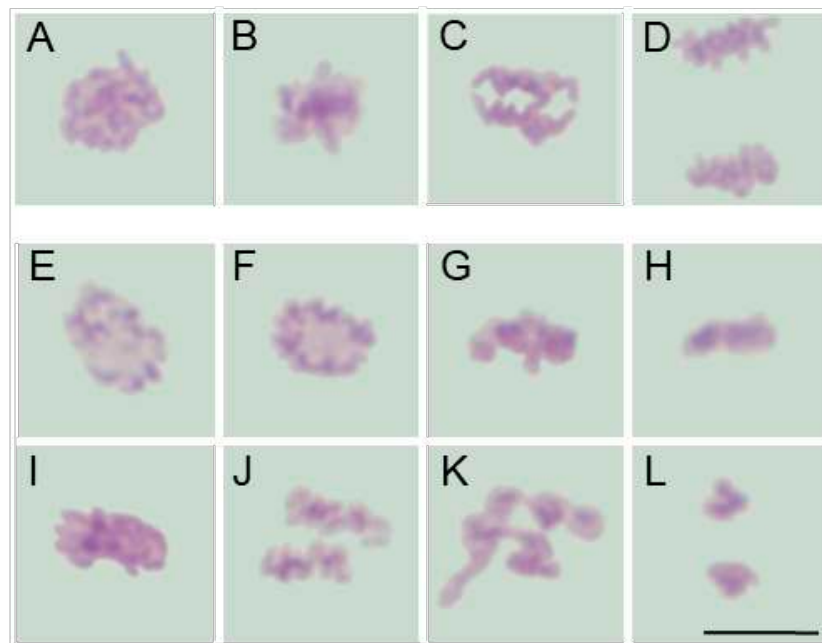


Figure 5. Mitotic figures in root meristem cells of *A. thaliana* after 24 h seedling incubation in water—Control (prophase: (A); metaphase: (B); anaphase: (C); telophase: (D)) and in emulsified SEO at the IC₅₀ concentration (prophase with uneven chromosome condensation and Feulgen-positive foci: (E,F); metaphase with excessively condensed chromosomes: (G); metaphase with insufficiently condensed chromosomes: (H); anaphase with sticky telomeric ends: (I); anaphase with uneven chromosome condensation: (J); anaphase with chromosomal bridges: (K); telophase with atypical chromatin condensation: (L)). Scale bar: 5 μ m.

2.2. Assessment of the SEO Effects on Cell Cycle Regulator

The observed decrease in mitotic activity prompted an analysis of the level of a key mitotic regulatory protein, Cdc2, whose presence is maintained throughout the cell cycle, in contrast to cyclins that undergo periodic synthesis and degradation. This choice allowed the detection of the protein regardless of the cell cycle phase. Western blot analysis focused on representatives of the Fabaceae and Brassicaceae families revealed that Cdc2 protein levels in root meristem cells of *V. faba* and *B. napus* were similar in the control and emulsifier-treated seedlings, with relative band intensities of 9 and 8 [a.u.] for *V. faba* and 10 and 11 [a.u.] for *B. napus*, respectively. Exposure to SEO at the IC₅₀ concentration resulted in a marked decrease in Cdc2 abundance, with intensities reduced to 6 [a.u.] in *V. faba* and 7 [a.u.] in *B. napus*, indicating that the essential oil inhibited the accumulation of this key cell cycle regulator (Figure 6).

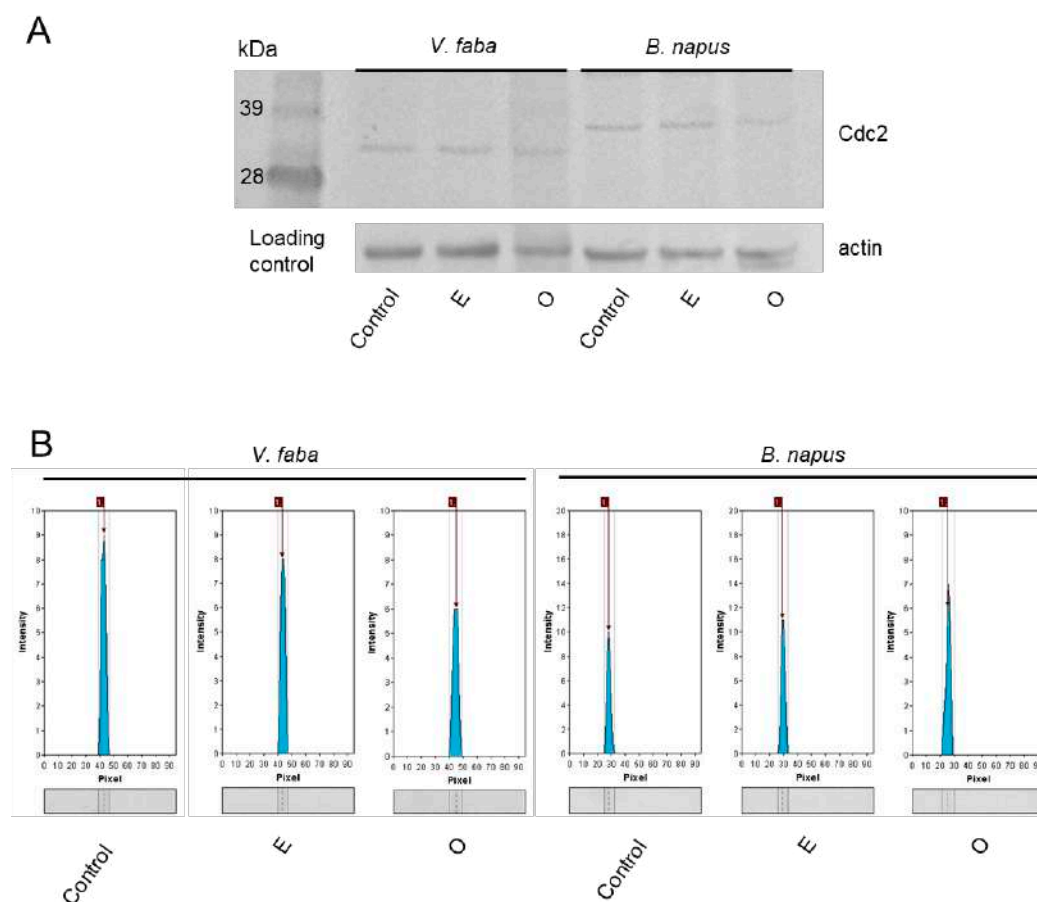


Figure 6. Western blot analysis of Cdc2 in protein extracts from the apical region of *V. faba* and *B. napus* roots after 24 h seedling incubation in water—Control, emulsifier solution—E, or emulsified SEO at the IC₅₀ concentration—O (A). Loading control represents the level of actin detected between 49 and 38 kDa. Microdensitometric analysis of Cdc2 band intensity on the membrane, performed using the GelAnalyzer software, to compare relative protein abundance between treatments (B).

2.3. Assessment of the SEO Effects on Plant Cytoskeleton

The observed disturbances in mitotic figures, particularly during metaphase and anaphase, suggested that defects in spindle organization might be responsible, as the dynamic reorganization of the mitotic spindle is essential for proper chromosome movement. Since the spindle is composed of polymerized heterodimers of α - and β -tubulin, which can be identified immunocytochemically, an analysis was undertaken to visualize β -tubulin using fluorescence microscopy.

In meristematic cells of control and emulsifier-treated roots of *V. faba* (Figure 7), *L. luteus* (Figure 8), *B. napus* (Figure 9), and *A. thaliana* (Figure 10), microtubules during interphase are organized into a cortical array composed of fine, delicate fibers (Figures 7–10(A–A')). In preprophase, they reorganize to form the preprophase band, a transient structure that marks the future division plane (Figures 7–10(B–B')). As this band disappears, microtubule material becomes concentrated at opposite regions of the nuclear envelope, where dispersed microtubule-organizing centers serve as nucleation sites for spindle assembly. In prophase, following nuclear envelope breakdown, chromosomes encounter microtubules nucleated from these dispersed centers (Figures 7–10(B–B'), C–C')). These microtubules, attached to kinetochores, form visible kinetochore fibers that align chromosomes at the metaphase plate and interlink to establish the spindle apparatus (Figures 7–10(D–D')). During anaphase, spindle microtubules depolymerize, enabling chromatid separation toward the poles, while sliding forces supported by polar microtubules in the central spindle contribute to pole separation (Figures 7–10(E–E')). Once chromatid segregation is completed in late anaphase (Figures 7–10(F–F')), remnants of the mitotic spindle give rise to the phragmoplast, a cytokinetic spindle (Figures 7–10(G–G')), which serves as a scaffold for cell plate formation. Seedlings treated with emulsifier showed no alterations in tubulin architecture compared to the control.

After 24 h of SEO treatment, the tubulin architecture in *V. faba* (Figure 11), *L. luteus* (Figure 12), *B. napus* (Figure 13), and *A. thaliana* (Figure 14) was clearly disturbed, with comparable abnormalities observed in all tested species. In interphase cells, cortical microtubule fibers appeared noticeably thicker and more intensely fluorescent than the delicate, finely dispersed microtubule arrays seen in control cells, suggesting stronger lateral associations and enhanced stabilization (Figures 11–14(A–A')). In preprophase cells (Figures 11–14(B–B')), the preprophase band also showed a more robust, sometimes distorted structure, often persisting unusually long into late prophase without effectively providing building material for spindle formation at the poles (see also Figures 11–14(C–C')). In metaphase, the kinetochore fibers within the forming spindle appeared densely packed, consisting of tightly bundled microtubules anchored at the kinetochores, forming conspicuously thick strands (Figures 11–14(D–D')). During anaphase, especially in *V. faba*, polar microtubules were clearly visible, displaying the same massive, bundled organization, with distinct tubulin accumulations near the kinetochores already pulled toward the poles (Figures 11–14(E–E')). In telophase, the architecture of the phragmoplast was similarly affected, with prominent, robust microtubule bundles forming massive strands within the cytokinetic spindle (Figures 11–14(F–F'), G–G')).

Another equally important component of the plant cell cytoskeleton is the actin network, which is highly responsive to various microlocal signals. In meristematic cells, the actin architecture undergoes dynamic reorganization, ranging from delicate, extended filaments forming a dispersed cortical network to more compact, locally concentrated bundles. In control cells and those treated with the emulsifier, both types of actin organization were observed in all four analyzed species (Figure 15(A–A'), C–C', E–E', G–G')). After 24-hour exposure of *V. faba* (Figure 15(B–B')), *L. luteus* (Figure 15(D–D')), *B. napus* (Figure 15(F–F')), and *A. thaliana* (Figure 15(H–H')) seedlings to SEO at the IC₅₀ concentration, a similar overall pattern was maintained; however, both the extended filaments and the compact bundles appeared thicker and more pronounced, exhibiting stronger fluorescence signals as a result.

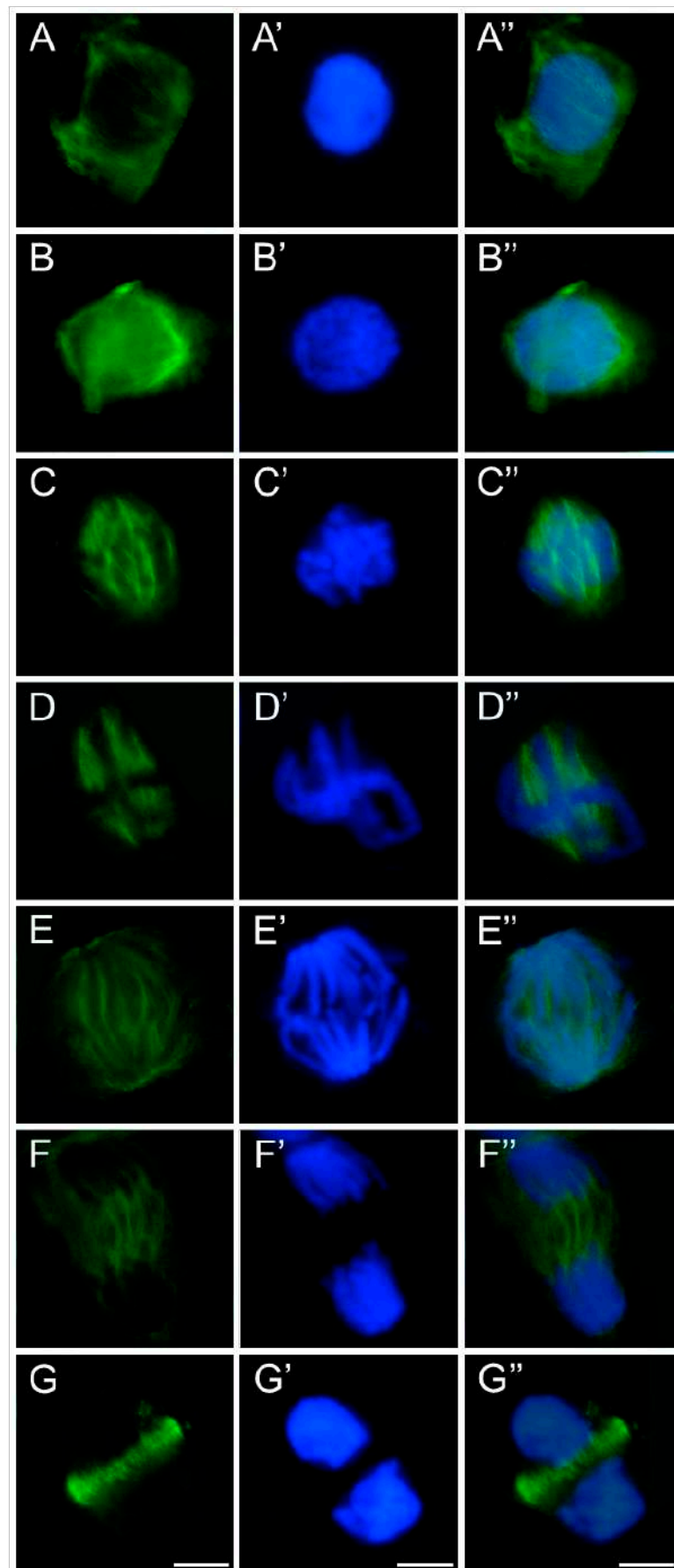


Figure 7. Immunocytochemical detection of β -tubulin (A–G) in root meristem cells of *V. faba* after 24 h seedling incubation in water—Control. Nuclei stained with DAPI (A'–G'), merged images (A''–G''). Scale bar: 10 μ m.

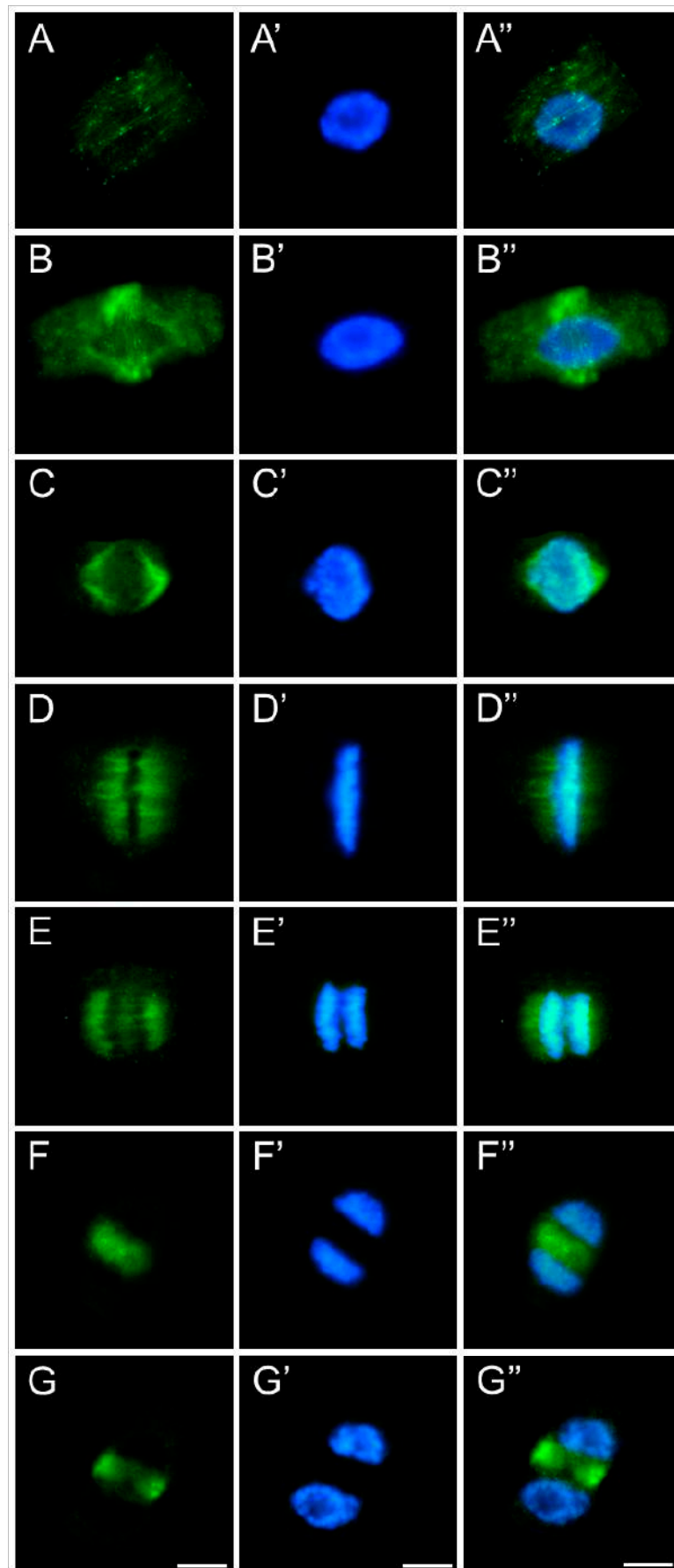


Figure 8. Immunocytochemical detection of β -tubulin (A–G) in root meristem cells of *L. luteus* after 24 h seedling incubation in water—Control. Nuclei stained with DAPI (A'–G'), merged images (A''–G''). Scale bar: 10 μ m.

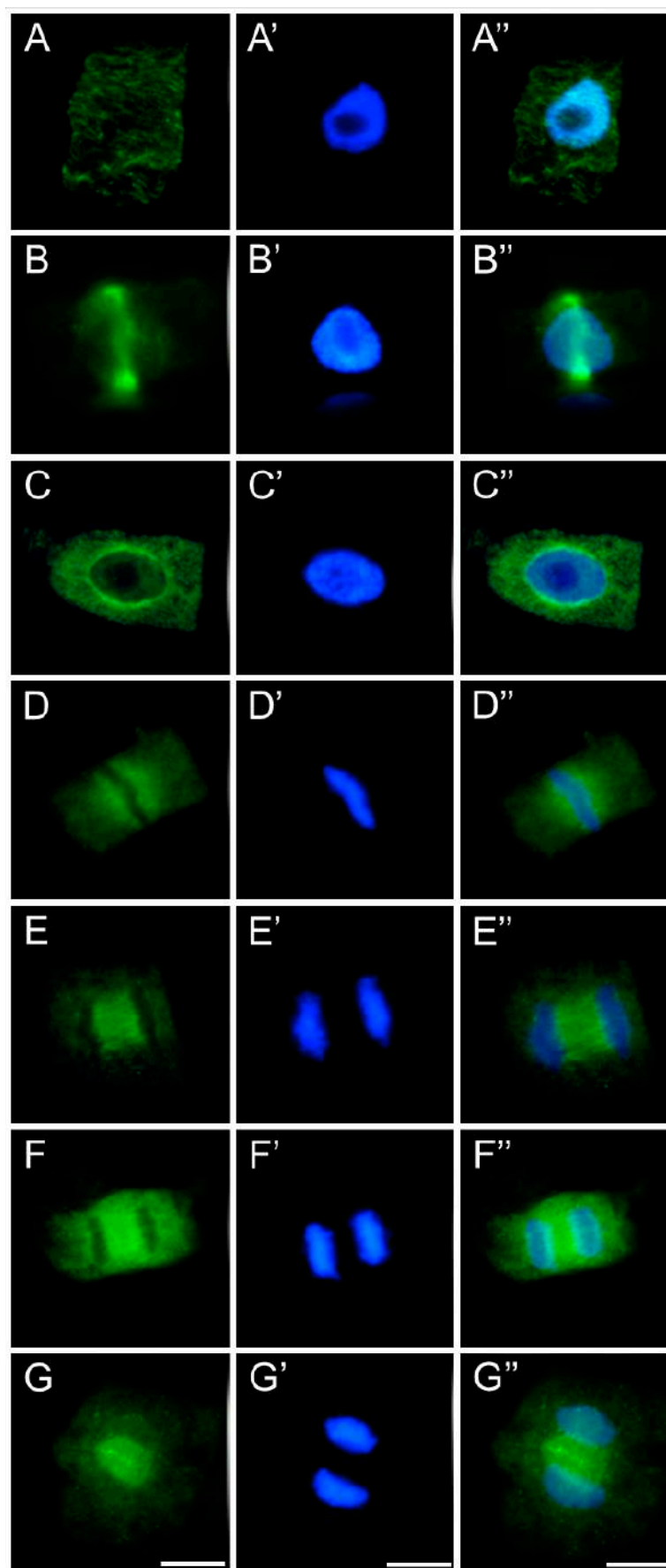


Figure 9. Immunocytochemical detection of β -tubulin (A–G) in root meristem cells of *B. napus* after 24 h seedling incubation in water—Control. Nuclei stained with DAPI (A'–G'), merged images (A''–G''). Scale bar: 10 μ m.

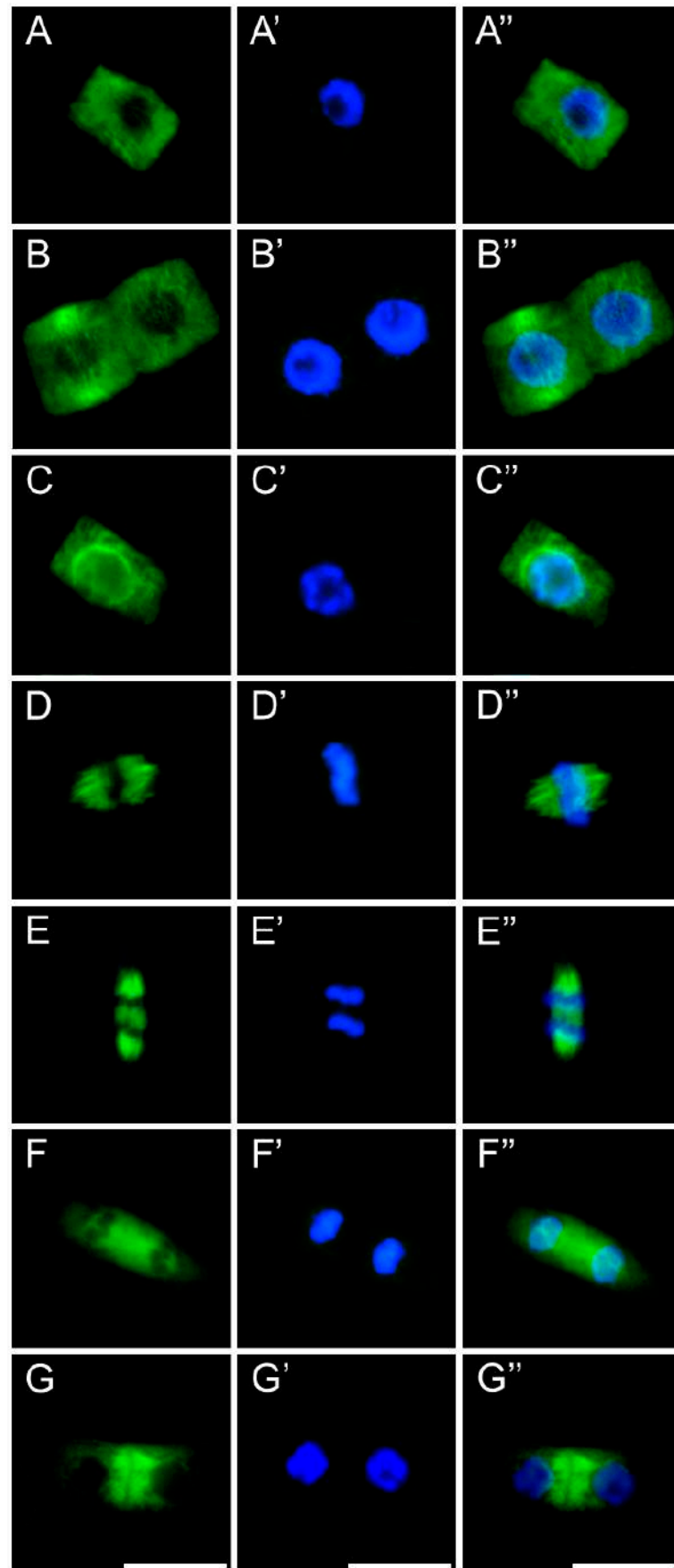


Figure 10. Immunocytochemical detection of β -tubulin (A–G) in root meristem cells of *A. thaliana* after 24 h seedling incubation in water—Control. Nuclei stained with DAPI (A'–G'), merged images (A''–G''). Scale bar: 10 μ m.

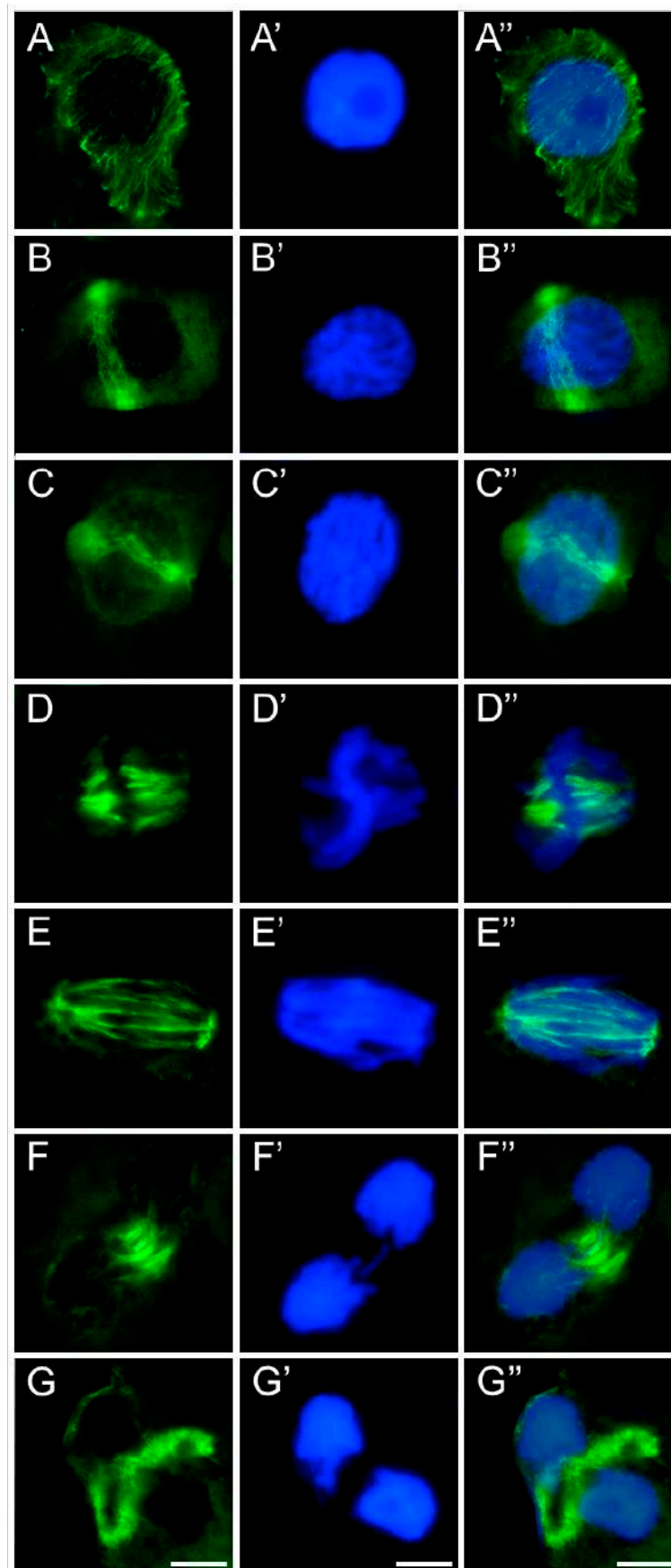


Figure 11. Immunocytochemical detection of β -tubulin (A–G) in root meristem cells of *V. faba* after 24 h seedling incubation in emulsified SEO at the IC_{50} concentration. Nuclei stained with DAPI (A'–G'), merged images (A''–G''). Scale bar: 10 μ m.

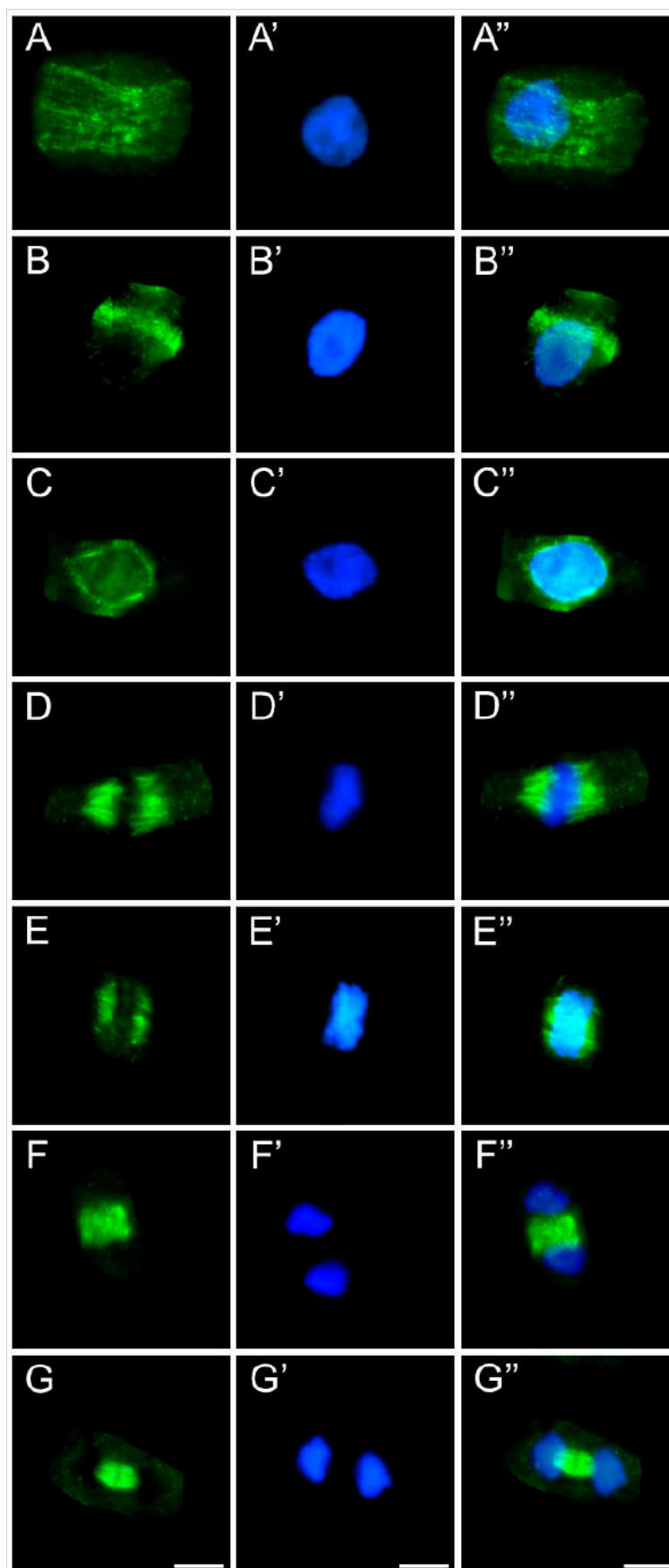


Figure 12. Immunocytochemical detection of β -tubulin (A–G) in root meristem cells of *L. luteus* after 24 h seedling incubation in emulsified SEO at the IC₅₀ concentration. Nuclei stained with DAPI (A'–G'), merged images (A''–G''). Scale bar: 10 μ m.

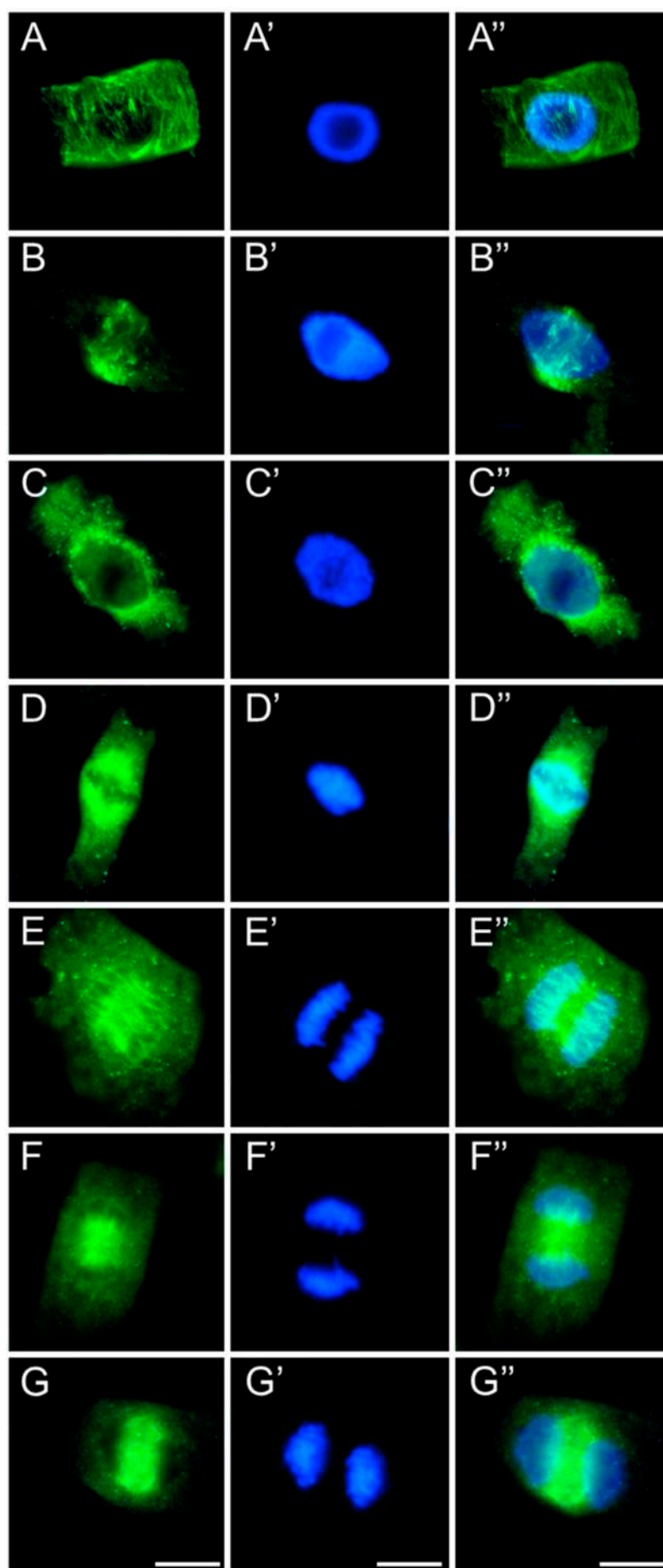


Figure 13. Immunocytochemical detection of β -tubulin (A–G) in root meristem cells of *B. napus* after 24 h seedling incubation in emulsified SEO at the IC₅₀ concentration. Nuclei stained with DAPI (A'–G'), merged images (A''–G''). Scale bar: 10 μ m.

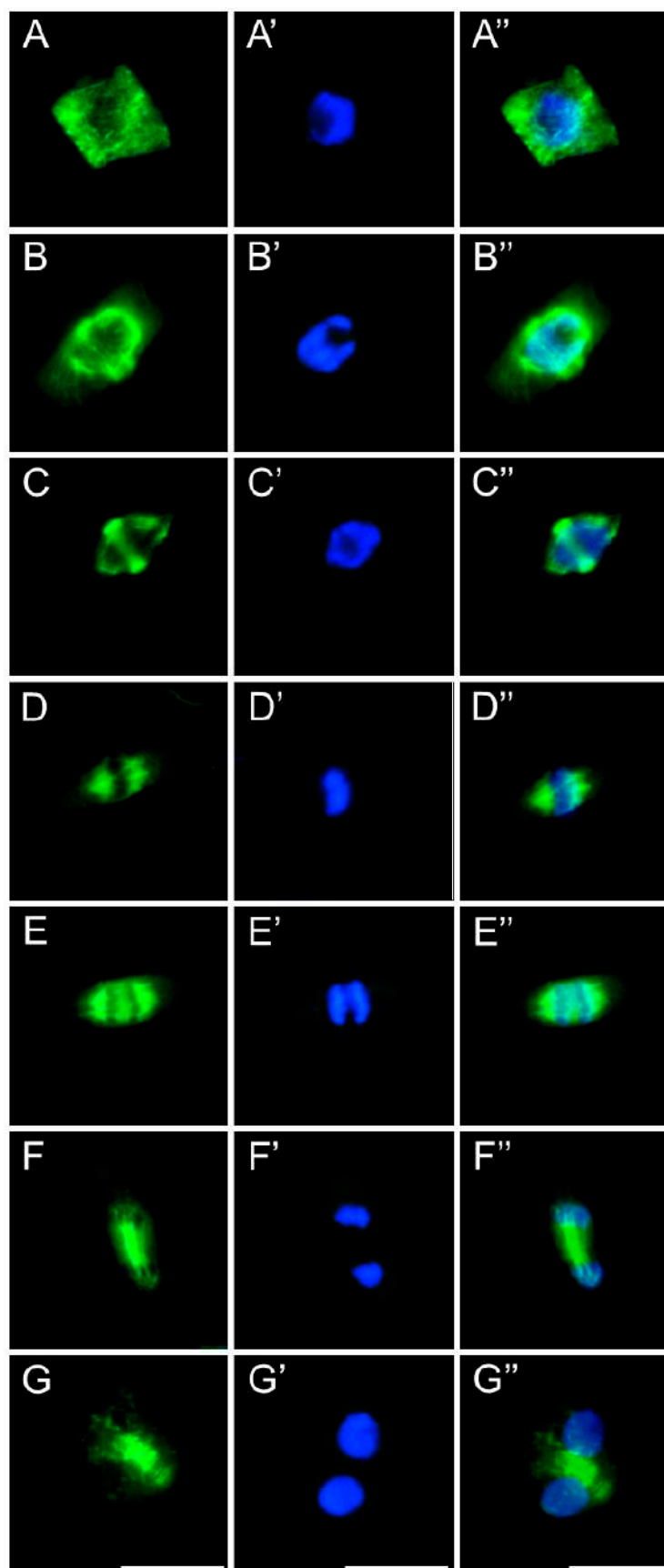


Figure 14. Immunocytochemical detection of β -tubulin (A–G) in root meristem cells of *A. thaliana* after 24 h seedling incubation in emulsified SEO at the IC₅₀ concentration. Nuclei stained with DAPI (A'–G'), merged images (A''–G''). Scale bar: 10 μ m.

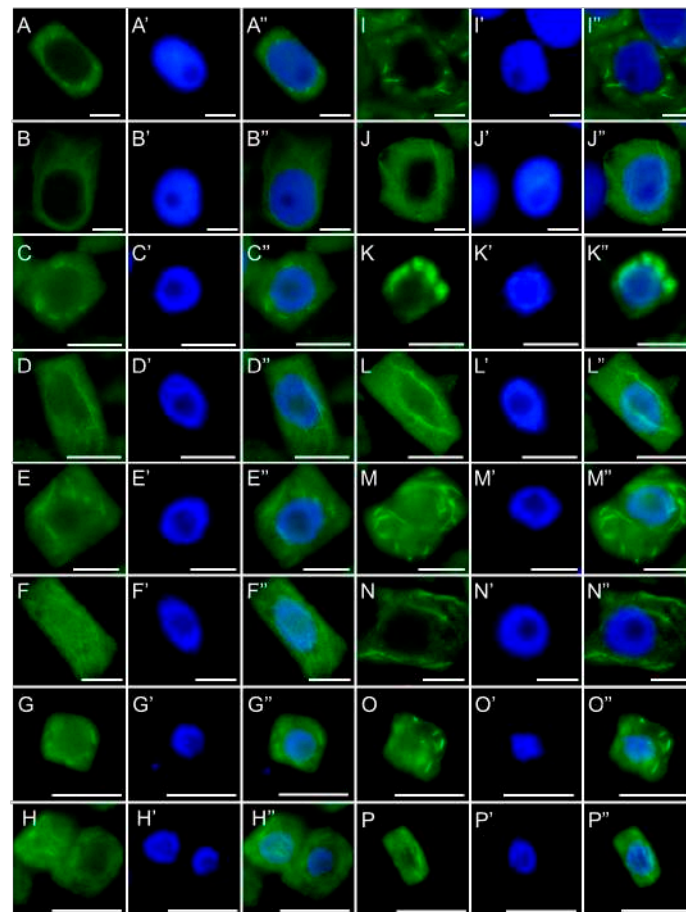


Figure 15. Immunocytochemical detection of actin (A–P) in root meristem cells of *V. faba* (A,B,I,J), *L. luteus* (C,D,K,L), *B. napus* (E,F,M,N), *A. thaliana* (G,H,O,P) after 24 h seedling incubation in water—Control (A–H) and in emulsified SEO at the IC₅₀ concentration (I–P). Panel descriptions are provided in the main text. Nuclei stained with DAPI (A'–P'), merged images (A''–P''). Scale bar: 10 μ m.

2.4. Assessment of the Alterations in Chromatin Structure by Modifying H3 Phosphorylation Patterns Followed by SEO Treatment

The condensation of chromatin into mitotic chromosomes and the recruitment of specific mitotic regulatory proteins are fundamental events ensuring the accurate segregation of genetic material during mitosis. This process is tightly regulated by specific histone epigenetic modifications. Among the most common epigenetic marks associated with mitosis are the phosphorylation of histone H3 at threonine 3 and serine 10. In all examined species, similar defects in chromatin condensation and mitotic figure morphology were observed. Therefore, the epigenetic analyses focused on *V. faba* as a representative of the Fabaceae family and *B. napus* as a representative of the Brassicaceae.

In mitotic root cells of control plants (*V. faba* and *B. napus*), the phosphorylated form of histone H3 at threonine 3 (H3T3Ph) appeared on early prophase chromosomes as discrete immunopositive foci (Figures 16 and 17(A–A'')). The number and intensity of these foci progressively increased as chromatin condensation advanced, culminating in a strong pericentromeric signal during late prophase, accompanied by weaker labeling along the chromosome arms (Figures 16 and 17(B–B'')). Upon chromosome alignment at the metaphase plate, the intensely labeled centromeric regions were positioned at the central plane, while the distinctly condensed metaphase chromosomes displayed a mosaic labeling pattern along the arms, with several foci of higher intensity, including enhanced fluorescence at the telomeric regions. However, due to interspecies differences in chromo-

some number and morphology, particularly in chromosome length, the overall labeling pattern in prophase and metaphase appeared slightly different between *V. faba* and *B. napus* (Figure 16(C–C'',D–D'') and Figure 17(C–C'')). Once sister chromatids began to separate during anaphase, the H3T3Ph signal markedly diminished in *V. faba* (Figure 16(E–E'')) and disappeared entirely in *B. napus* (Figure 17(D–D'')), leaving telophase chromosomes devoid of specific detectable labeling (Figure 16(F–F'') and Figure 17(E–E'')).

Following treatment with SEO at the IC₅₀ concentration, H3T3Ph-associated fluorescence signals were generally markedly enhanced in mitotic cells of both *V. faba* and *B. napus*, with increased signal intensity and expanded distribution of immunopositive foci (Figure 16(G–P,G'–P',G''–P''),R) and Figure 17(F–I,F'–I',F''–I''),K)). However, the mitotic cell population displayed heterogeneous chromatin condensation patterns in particular phases, which corresponded to variable fluorescence labeling profiles (especially evident in *V. faba* due to large chromosomes, as shown in the panel; similar effects were seen in *B. napus*, but not imaged). In metaphase cells with strongly condensed chromosomes, intense fluorescence was observed along the chromosome arms, often with enhanced signals at telomeric regions (Figure 16(J–J'')). In contrast, cells exhibiting atypically low condensation of metaphase chromosomes showed a more diffuse and extended labeling pattern, lacking the characteristic pericentromeric enrichment seen in control cells, and displaying a markedly reduced mosaic signal along the arms and telomeres (Figure 16(K–K'',L–L'')). Moreover, in both species, while structurally normal anaphases were observed, a subset of cells exhibiting structural abnormalities during anaphase showed persistent strong H3T3Ph labeling into early anaphase (Figure 16(M–M'')). In later stages of anaphase, this signal was weaker and primarily restricted to the pericentromeric regions or remained visible along the arms of the chromosomes (Figure 16(N–N'',O–O'')). During telophase, residual H3T3Ph labeling was occasionally detectable as small discrete foci distributed along the chromosome arms in a fraction of cells (compare Figure 16(P–P'',Q–Q'')).

Under physiological conditions, the phosphorylated form of histone H3 at serine 10 (H3S10Ph) is predominantly localized to the pericentromeric regions of mitotic chromosomes (Figures 18 and 19(A–D,A'–D',A''–D'')). The signal first appears in early prophase, typically as intensely fluorescent foci clustered at one pole of the nucleus. In species with a high DNA content, such as *Vicia faba*, this distribution reflects the classical Rabl configuration, in which chromosomes maintain a polarized orientation inherited from the previous mitotic division (Figure 18(A–A'')). In contrast, in species with lower DNA content, such as *Brassica napus*, the H3S10Ph signal may be more evenly distributed across the entire prophase nucleus, without clear polarization (Figure 19(A–A'')). In metaphase, the fluorescent signal aligns along the metaphase plate, corresponding to the position of centromeres arranged in the division plane (Figures 18 and 19(B–B'')). During anaphase, the labeled centromeric regions migrate toward opposite poles, consistent with the movement of centromeres being pulled first by the spindle microtubules (Figures 18 and 19(C–C'')). In telophase, as chromatin begins to decondense, the intensity of the H3S10Ph signal gradually diminishes—remaining faintly detectable in some cells, but typically disappearing entirely in later stages (Figures 18 and 19(D–D'')). This characteristic labeling pattern was observed in both control cells and cells treated with the emulsifier in root meristems of *V. faba* and *B. napus*.

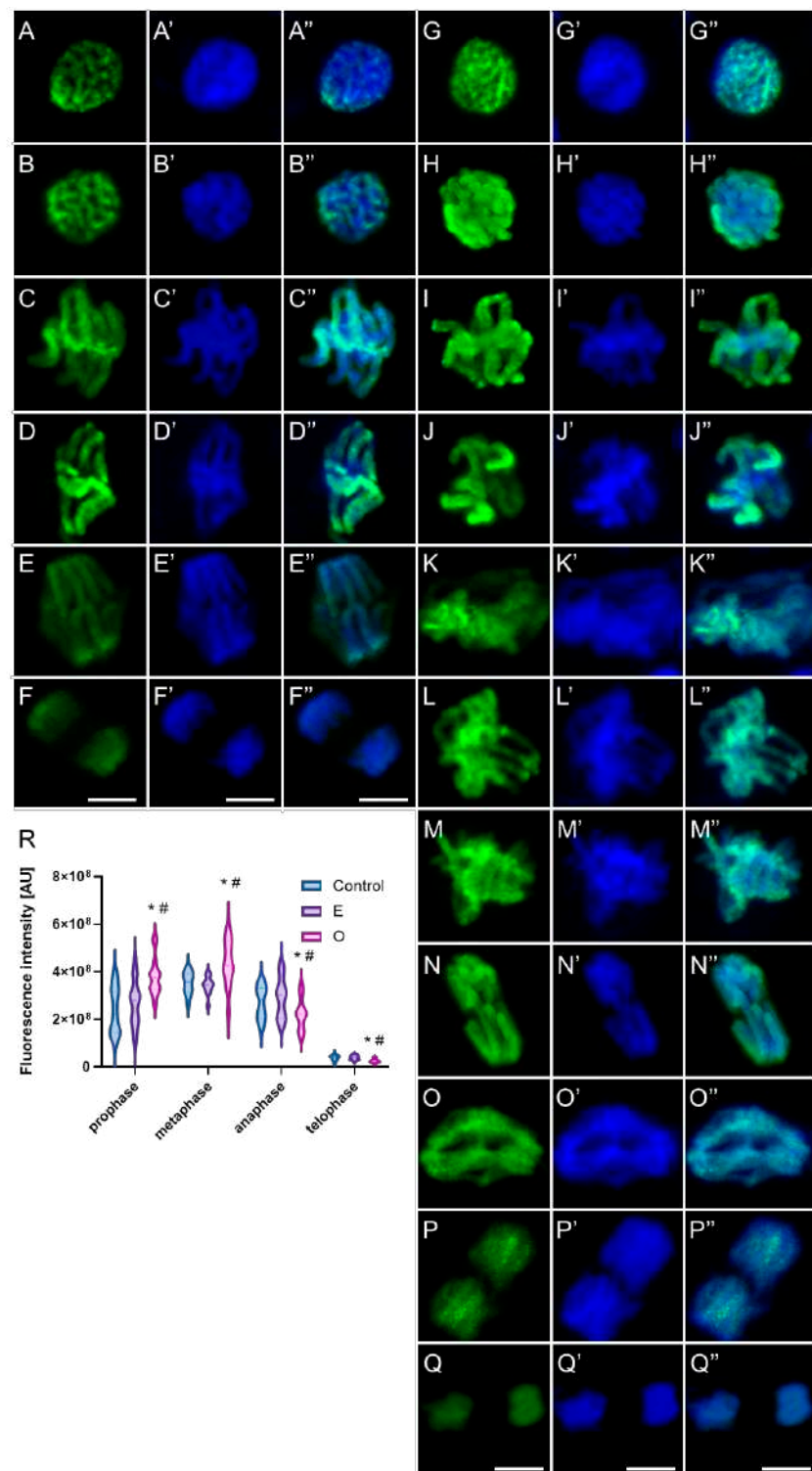


Figure 16. Immunofluorescence detection of H3T3Ph in root meristem cells of *V. faba* after 24 h seedling incubation in water—Control (A–F) and in emulsified SEO at the IC₅₀ concentration (G–Q). Nuclei stained with DAPI (A'–Q'), merged images (A''–Q''). Panel descriptions are provided in the main text. Scale bar: 10 μ m. Mean fluorescence intensity of the H3T3Ph signal in prophase, metaphase, anaphase and telophase root meristem cells of *V. faba* after 24 h seedling incubation in water—Control, the emulsifier solution—E, or emulsified SEO at the IC₅₀ concentration—O, (R). The width of each violin plot represents the distribution of values along the y-axis; medians are indicated by dashed lines, and quartiles by dotted lines. Statistical differences were assessed using the Mann–Whitney test at $p \leq 0.05$. Asterisk (*) and hash (#) marks denote significant differences compared to the control and the emulsifier, respectively.

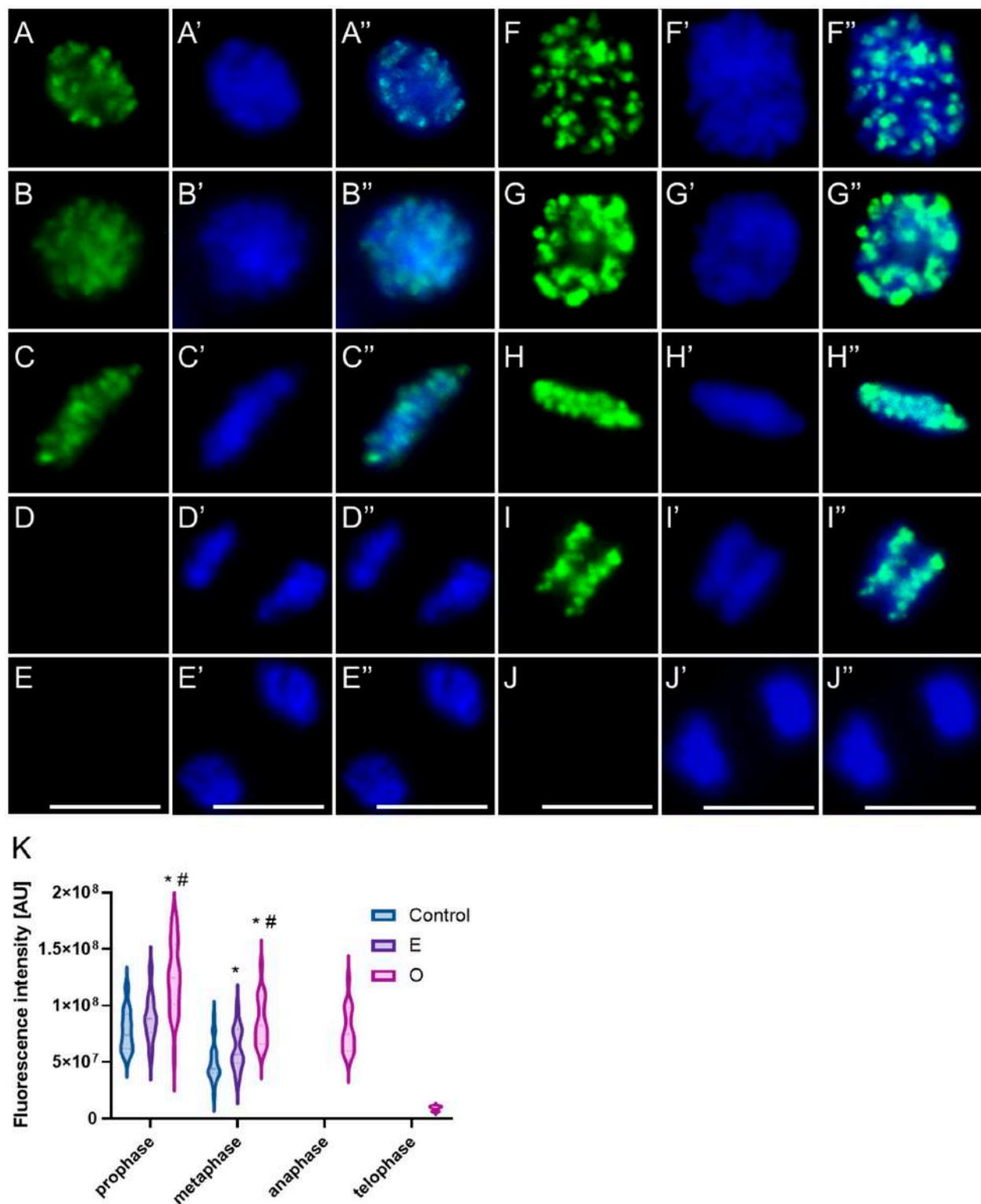


Figure 17. Immunofluorescence detection of H3T3Ph in root meristem cells of *B. napus* after 24 h seedling incubation in water—Control (A–E) and in emulsified SEO at the IC₅₀ concentration (F–J). Nuclei stained with DAPI (A'–J'), merged images (A''–J''). Panel descriptions are provided in the main text. Scale bar: 10 μ m. Mean fluorescence intensity of the H3T3Ph signal in prophase, metaphase, anaphase and telophase root meristem cells of *B. napus* after 24 h seedling incubation in water—Control, the emulsifier solution—E, or emulsified SEO at the IC₅₀ concentration—O, (K). The width of each violin plot represents the distribution of values along the y-axis; medians are indicated by dashed lines, and quartiles by dotted lines. Statistical differences were assessed using the Mann–Whitney test at $p \leq 0.05$. Asterisk (*) and hash (#) marks denote significant differences compared to the control and the emulsifier, respectively.

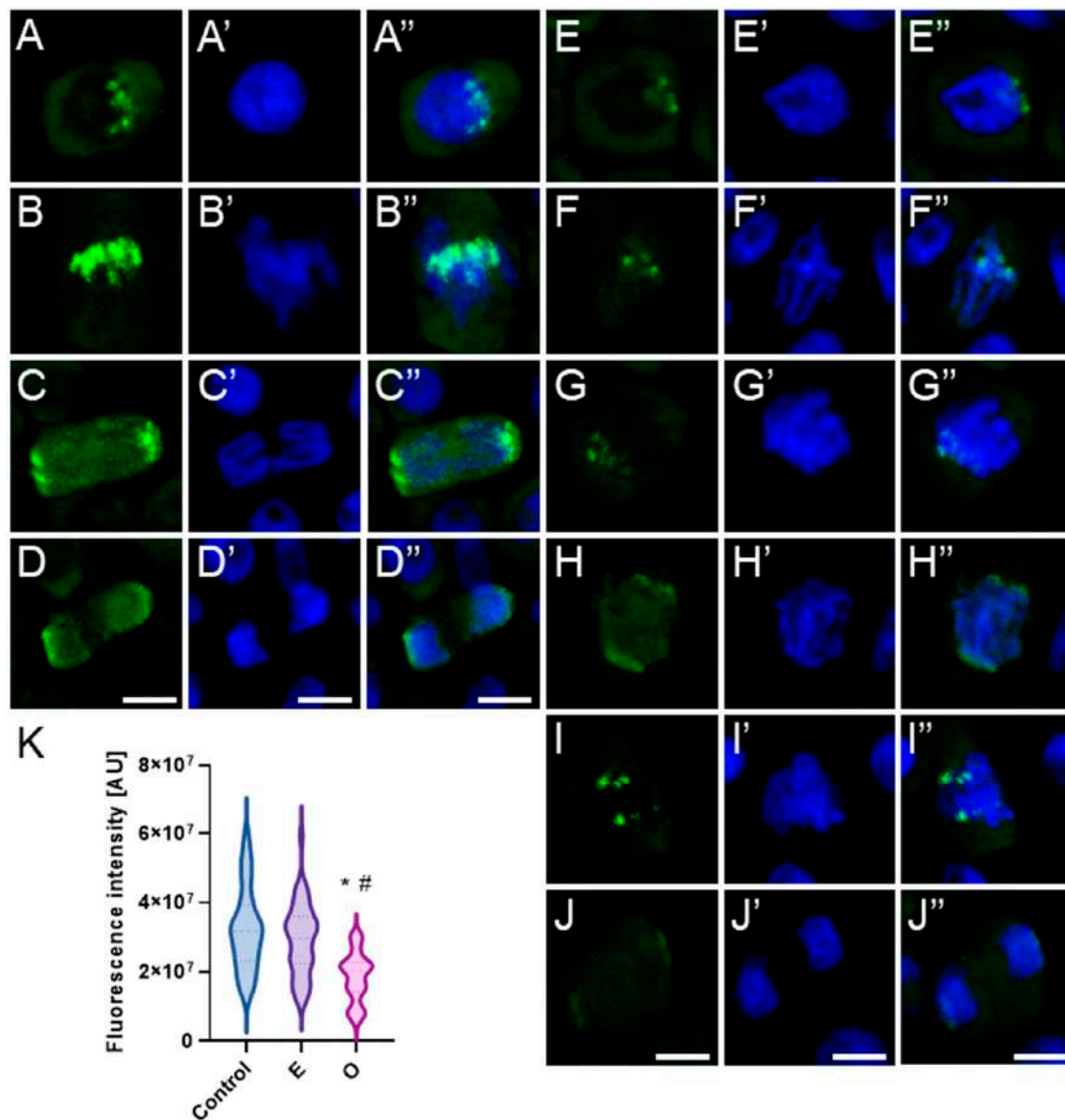


Figure 18. Immunofluorescence detection of H3S10Ph in root meristem cells of *V. faba* after 24 h seedling incubation in water—Control (A–D) and in emulsified SEO at the IC₅₀ concentration (E–J). Nuclei stained with DAPI (A'–J'), merged images (A''–J''). Panel descriptions are provided in the main text. Scale bar: 10 μ m. Mean fluorescence intensity of the H3S10Ph signal in prophase, metaphase, anaphase and telophase root meristem cells of *V. faba* after 24 h seedling incubation in water—Control, the emulsifier solution—E, or emulsified SEO at the IC₅₀ concentration—O (K). The width of each violin plot represents the distribution of values along the y-axis; medians are indicated by dashed lines, and quartiles by dotted lines. Statistical differences were assessed using the Mann–Whitney test at $p \leq 0.05$. Asterisk (*) and hash (#) marks denote significant differences compared to the control and the emulsifier, respectively.

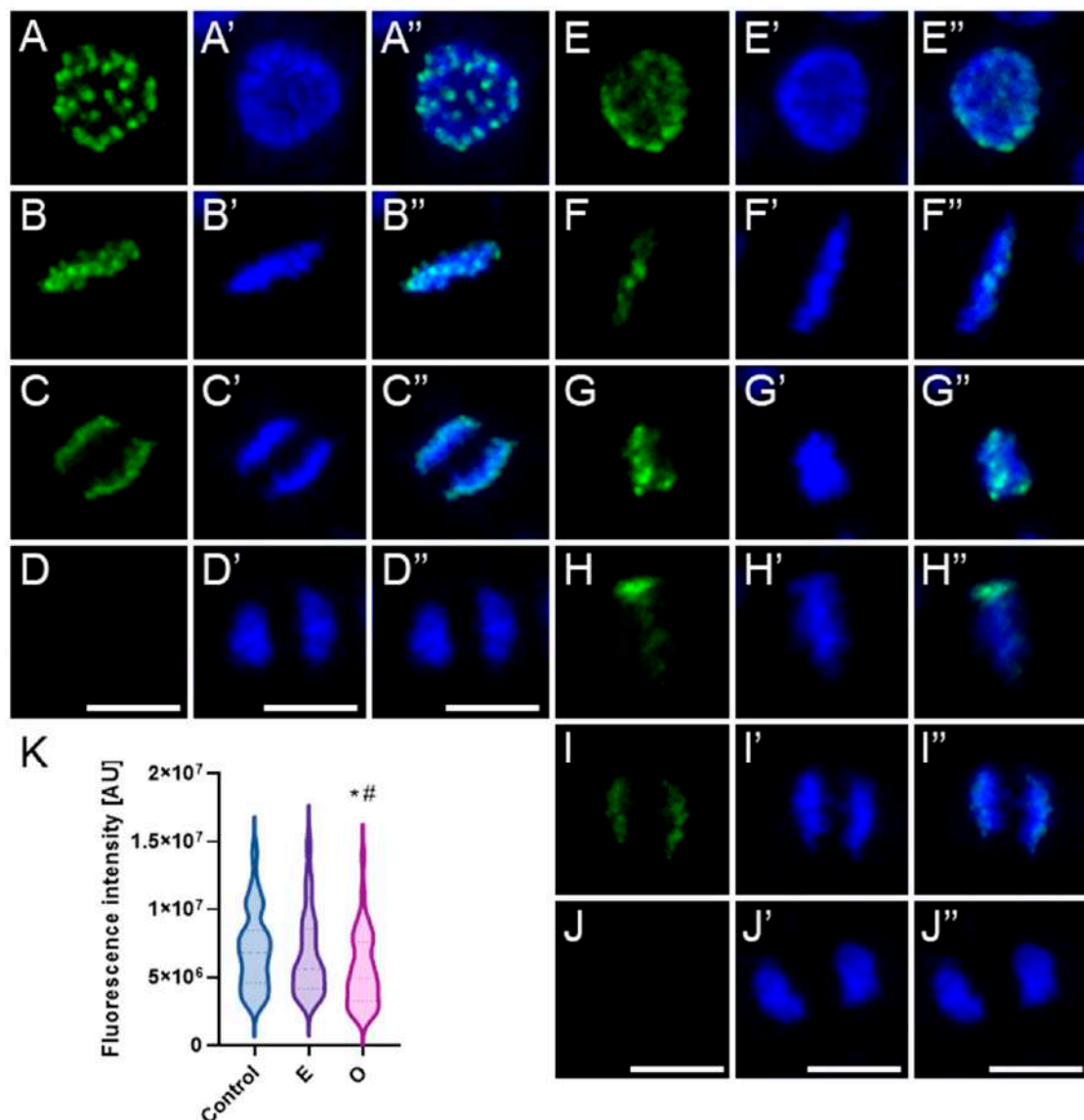


Figure 19. Immunofluorescence detection of H3S10Ph in root meristem cells of *B. napus* after 24 h seedling incubation in water—Control (A–D) and in emulsified SEO at the IC₅₀ concentration (E–J). Nuclei stained with DAPI (A'–J'), merged images (A''–J''). Panel descriptions are provided in the main text. Scale bar: 10 μ m. Mean fluorescence intensity of the H3S10Ph signal in prophase, metaphase, anaphase and telophase root meristem cells of *B. napus* after 24 h seedling incubation in water—Control, the emulsifier solution—E, or emulsified SEO at the IC₅₀ concentration—O (K). The width of each violin plot represents the distribution of values along the y-axis; medians are indicated by dashed lines, and quartiles by dotted lines. Statistical differences were assessed using the Mann–Whitney test at $p \leq 0.05$. Asterisk (*) and hash (#) marks denote significant differences compared to the control and the emulsifier, respectively.

Following treatment with SEO at the IC₅₀ concentration, no changes in the localization of the H3S10Ph signal were observed in root meristematic cells of either species (Figures 18 and 19(E–J, E'–J', E''–J'')). Fluorescent foci appeared in the same chromosomal regions as under control conditions; however, the overall signal intensity was markedly reduced in *V. faba* and slightly less so in *B. napus* (Figures 18K and 19K). Moreover, metaphase and early anaphase chromosomes exhibiting exceptionally strong condensation emitted a more intense fluorescence signal (Figure 18(I–I'') and Figure 19(G–G'')), whereas in metaphase chromosomes with atypically weak condensation, the H3S10Ph signal was faint or even undetectable (Figure 18(G–G'') and Figure 19(H–H'')).

3. Discussion

Plant growth, from the earliest stages of development through to the end of the life cycle, is sustained by the continuous activity of meristematic tissues. Consequently, cell cycle is at the core of current research efforts aimed at identifying effective strategies to suppress the growth of undesirable plants, thereby enhancing the protection and productivity of crop species in monoculture systems [41]. In response to the growing demand for environmentally safe plant protection agents, increasing attention has been directed toward natural product-based alternatives [25,28,32,95,96]. In our study, which focused on evaluating the bioherbicidal potential of essential oil extracted from the rhizomes of sweet flag (*A. calamus*, SEO), we also investigated its impact on the progression of the plant cell cycle.

3.1. Bioherbicidal Potential of SEO: Root Growth Inhibition via Cell Cycle Disruption

Our previous research demonstrated that SEO exerts a species-specific, concentration-dependent phytotoxic effect, limiting Brassicaceae growth while sparing Fabaceae. This effect was associated with oxidative stress: Fabaceae accumulated higher ROS levels but activated more efficient antioxidant defenses, whereas Brassicaceae, despite lower ROS accumulation, experienced stronger metabolic disruptions [22]. In root meristems, this oxidative stress triggered replication stress, causing DNA double-strand breaks. Consequently, the replication index dropped to 70% of control levels in Fabaceae and 80% in Brassicaceae, and replication dynamics decreased to 80% and 70% of control, respectively. While root meristem cells exhibited a unified pattern of response to SEO-induced stress, their sensitivity varied across species. These early effects on the cell cycle were followed, as confirmed in the present study, by disruptions in mitotic progression (Figure 20). SEO significantly reduced cell proliferation, as reflected by a 50–60% decrease in the mitotic index, promoted the accumulation of cells at metaphase, and induced mitotic aberrations, including irregular chromatin organization and structural defects in karyokinetic and cytokinetic spindles. The reduction in mitotic index may partly result from the accumulation of cells in G1, the reduced proportion of cells entering S phase, and impaired replication dynamics associated with DNA damage, including difficulties in replicating highly condensed heterochromatin, as indicated by an increased proportion of cell in the late S phase [41]. However, the pronounced inhibition of mitotic activity and the structural abnormalities observed in dividing cells indicate multifaceted interference with key, interdependent mechanisms regulating mitosis. Notably, similar effects, specifically a decrease in mitotic activity, were reported by Rajamanikkam et al. [42] in *Allium cepa* root tips treated with an aqueous extract of *A. calamus*, although without observable chromosomal abnormalities. Given the complex composition of SEO, as characterized by others [81] and in our previous study [91], including major and minor constituents that may act synergistically, it remains challenging to attribute these effects to specific compounds. Nevertheless, the high content of phenylpropanoids, particularly asarones, known for their strong biological activity, is likely to contribute substantially to the observed phytotoxic and cell cycle–modulating effects [40].

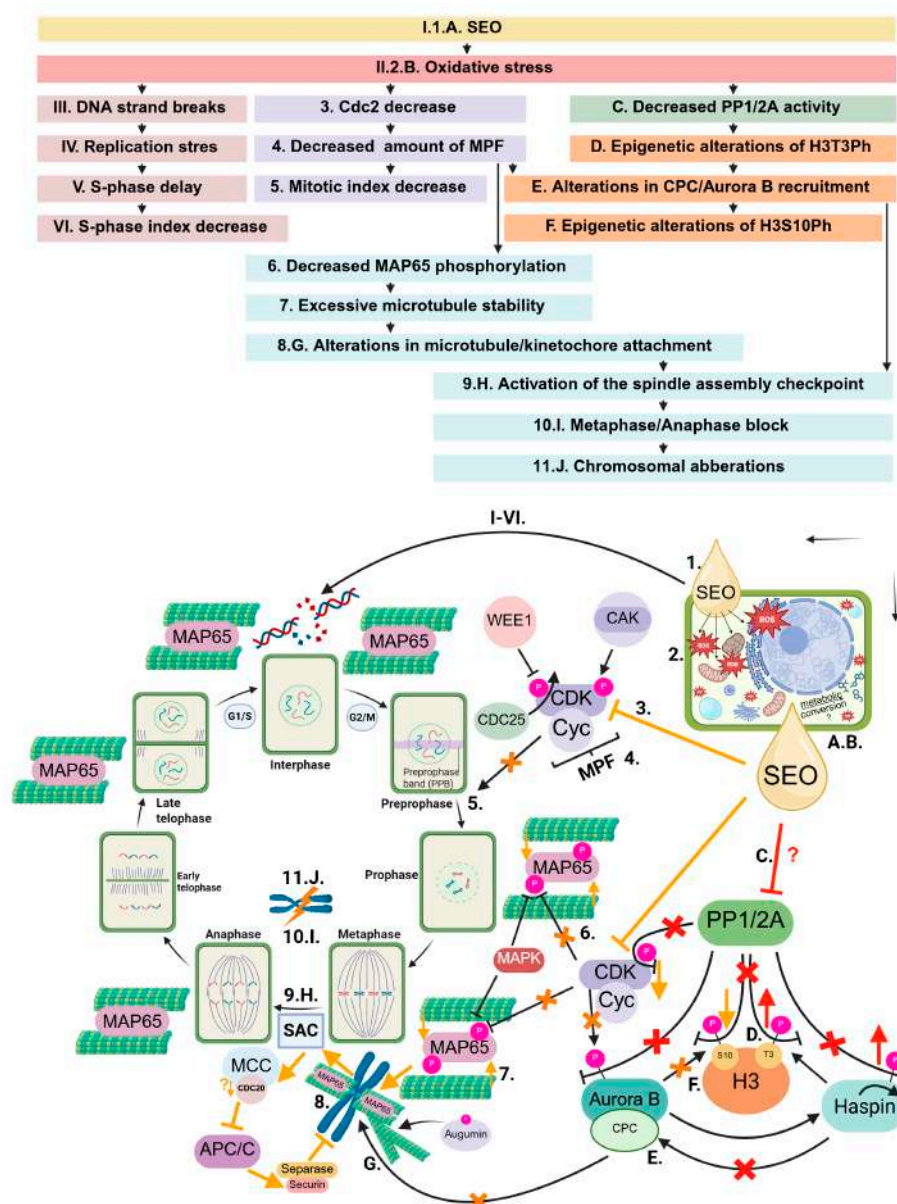


Figure 20. Effect of SEO on meristematic cell proliferation: ROS generation and DNA break formation lead to a slowdown of S-phase progression (pathways I–VI), mitotic entry arrest, and mitotic defects through decreased levels of cyclin-dependent kinase Cdc2 (the conserved plant CDKA homolog) and alterations in microtubule dynamics (pathways 1–11), as well as H3 histone epigenetic disturbances, likely associated with reduced PP1/2A phosphatase activity (pathways A–J). The pathway numbering used in the flowchart is maintained in the illustrative panel depicting the processes discussed. A detailed description of the events is provided in the Introduction and Discussion, and abbreviations are explained in the “Abbreviations” section. Orange arrows indicate changes resulting from cyclin-dependent kinase deficiency, with orange X marks representing the processes affected. Red arrows and X marks similarly denote effects of PP1/2A activity inhibition. Created with biorender.com.

3.2. Underlying Mechanisms of Mitotic Index Reduction

Our previous studies revealed that SEO, aside from asarones, consists of a number of phenylpropanoid compounds, including methyl-eugenol, safrol, isoacoramone, and asaronaldehyde [97]. However, comprehensive studies examining the effects of these compounds across both plant and animal models remain limited, and the existing data do not yet provide a complete picture of their biological activity. In the present study, Western blot analysis revealed that SEO treatment led to a noticeable decrease in the abundance of

the cell cycle regulator CDKA, detected using anti-Cdc2 antibodies (the conserved homolog of plant CDKA) in root apices of both *V. faba* and *B. napus*. Given that Cdc2 protein levels are generally maintained at a relatively constant level throughout the cell cycle, unlike cyclins whose abundance fluctuates, the observed reduction suggests either enhanced proteolytic degradation or decreased synthesis of this key mitotic kinase.

It is well established that phenylpropanoid compounds strongly affect plant cell cycle regulation. For instance, in *A. thaliana*, eugenol was shown to inhibit growth by upregulating stress-response genes while downregulating those required for mitotic progression, including CYCB which promotes entry into mitosis, TPX2 involved in microtubule organization and spindle assembly, and CDC20.1 required for sister chromatid separation [98,99]. Eugenol also suppressed genes involved in the biosynthesis of cell wall components (lignin, cellulose, hemicellulose, and pectin), essential for proper cell plate formation during cytokinesis [99]. Phenyl carboxylic derivatives of phenylpropanoids, such as benzoic and cinnamic acid derivatives, similarly inhibited root growth in germinating cucumber seeds by downregulating key cell cycle genes, including CDKA, CDKB, and several cyclins (CycA, B, D3;1, D3;2) [100]. Additional evidence for the antiproliferative potential of phenylpropanoids comes from studies on cinnamaldehyde and its analogs, which induced G2 arrest in human cancer cells by downregulating key G2/M regulators (CDK1, CDC25C, MAD2, CDC20). This was accompanied by CycB1 accumulation, which in the absence of active CDK1 failed to trigger mitotic entry [101]. Although the direct impact of asarones on gene expression and regulatory mechanisms in dividing plant meristematic cells remains unclear, their antiproliferative activity is well established in cancer models [35]. For example, Li et al. [102] demonstrated that β -asarone induces G1 arrest in glioma cells by upregulating the CKIs (p21 and p27), while downregulating key activators such as CDC25A, CDK2, Cyc D, and E.

These findings provide a rationale to suggest that, given the universal nature of the core regulatory mechanisms governing the cell cycle across plant, animal, and even cancer cells, the components of SEO may contribute to broad changes in gene expression in meristematic cells, beyond the observed reduction in Cdc2 (CDKA) abundance (Figure 20). The potential repression of cell cycle activators or the upregulation of inhibitors aligns with our observations—namely, the reduction in the proportion of mitotic cells, which could result from the failure to assemble an active mitosis-promoting factor (MPF), whose formation depends on the precise interaction of specific cyclins and kinases. At the same time, it is important to consider that SEO may interfere with the multistep activation of pre-existing CDK/cyclin complexes, a process that relies on the coordinated activities of WEE1 kinase, CAK (itself a cyclin—CDK complex), and CDC25-like phosphatases, which are further regulated by PP1 and PP2A phosphatases (Figure 20) [103]; SEO may potentially inhibit PP1/2A activity, as further discussed in relation to histone H3 epigenetic modifications. We are only at the early stage of uncovering the mechanism of SEO action, yet it is evident that it targets the intricate, multilayered regulatory network controlling cell proliferation, where disruption of even a single component may propagate through entire cascades of signaling events.

3.3. Disruption of Mitotic Progression via Cytoskeletal Disorganization Induced by SEO

A second clear target of SEO activity was the disruption of cells that had already passed the G2 checkpoint and entered division. The marked increase in the metaphase index indicates interference with spindle microtubule function, similar to the effects of classical microtubule-disrupting agents such as colchicine, oryzalin, and propyzamide [104].

This hypothesis was further supported by immunofluorescence analysis with anti-tubulin antibodies, which revealed major alterations in microtubule organization. Mi-

crotochubules appeared thickened, densely bundled, and formed distinct bands, suggesting excessive lateral association and stabilization within the spindle structure. Additionally, difficulties in microtubule reorganization were observed, including the prolonged persistence of the preprophase band, even though nuclear morphology indicated that cells had already entered prometaphase—a stage that, under control conditions, typically coincides with the rearrangement of the microtubule network around the disassembling nuclear envelope [83].

Similarly, immunolabeling of actin microfilaments revealed enhanced fluorescence, indicating thickening, excessive stabilization, or aggregation that could impair proper reorganization during mitosis under SEO treatment. Despite these alterations, the general architecture of the actin cytoskeleton remained preserved. Short, bundled microfilaments and long strands stabilizing the nucleus in interphase were still present, along with the preprophase ring, the cage-like structure around the metaphase spindle, and short filaments in the phragmoplast. One potential target of SEO action may be the plant actin-binding protein AtFormin14 (AtFH14), which in *A. thaliana* links microfilaments and microtubules, facilitating proper spindle organization [105]. It is noteworthy that disturbances in actin organization in response to phenylpropanoids have not yet been reported in plant cells, making definitive conclusions difficult. Some insights come from the study of Sarheed et al. [85], in which the effects of various mint essential oils on the cytoskeleton of *A. thaliana* were evaluated. The authors reported that *Agastache rugosa* oil caused microtubule degradation, while *Mentha longifolia* had no effect on microtubules but led to actin elimination. In earlier work [20], neither *A. rugosa* oil nor its constituents (menthone and isomenthone) eliminated actin filaments, but rather induced their bundling into thick cables. Since these compounds are monoterpenes, this highlights that cytoskeletal responses may vary considerably even within the same chemical class.

However, disruptions in microtubule organization involving tubulin aggregation were also observed by Hermes et al. [106] in human hepatocellular carcinoma cells (HepG2) exposed to asarones and their epoxide metabolites. Although the overall microtubule network remained intact, its spatial organization was clearly altered, leading to tubulin condensation. Similar findings were reported by Nagle et al. [101], who demonstrated tubulin aggregation in colorectal cancer cells (HCT 116) treated with cinnamaldehyde, another phenylpropanoid compound. As suggested by Guo et al. [99], such cytoskeletal abnormalities may result from the downregulation of genes encoding spindle-associated proteins, such as TPX2. However, the direct effect of SEO constituents on the activity of cytoskeleton-associated proteins involved in microtubule remodeling cannot be ruled out.

Since the mitotic spindle still formed, it is unlikely that SEO interfered with proteins responsible for microtubule nucleation, such as the γ -tubulin ring complex (γ -TuRC), kinesin-14 motor proteins transporting γ -TuRC to the spindle poles, or α -Aurora kinase, which phosphorylates γ -TuRC in a complex with TPXL3 to promote its spindle association [107]. Similarly, RUNKEL kinase, which contains a microtubule-binding domain and participates in phragmoplast expansion during cytokinesis, appears to remain active [108].

A likely target of SEO is the MAP65 family of microtubule-associated proteins, which mediate lateral bundling of microtubules into parallel arrays and stabilize the mitotic spindle (Figure 20) [109]. In *A. thaliana*, the MAP65-1 binds to microtubules during interphase, anaphase, and telophase, but not during prophase or metaphase. Its function is tightly regulated by CDK and MAPK kinases [83,109]. Phosphorylation of MAP65-1 inhibits its microtubule-binding, while lack of phosphorylation leads to excessive accumulation and stabilization of microtubules, particularly in the central region of the metaphase spindle, delaying anaphase onset [109]. It is therefore highly probable that SEO, by affecting the expression of genes encoding cyclins and CDK kinases or by interfering with their acti-

vation, could have impaired or significantly reduced MAP65 phosphorylation, resulting in its sustained activity throughout mitosis. Consequently, prolonged lateral bundling of microtubules and impaired spindle remodeling may have occurred (Figure 20). The formation of excessively dense microtubule bundles could also involve disruptions in augmin function, which mediates branched microtubule nucleation and dense network formation [86]. Although direct evidence in plants is lacking, by analogy with animal cells, augmin's interaction with microtubules may be regulated by phosphorylation via CDK or α -Aurora kinases [110]. These hypotheses will be tested experimentally in the next phase of our research.

Improperly formed, laterally bundled microtubule arrays may have hindered correct kinetochore attachment, potentially activating the spindle assembly checkpoint (SAC) at the metaphase/anaphase transition and sustaining activity of the mitotic checkpoint complex (MCC), which binds and inhibits CDC20, thereby preventing the activation of the anaphase-promoting complex/cyclosome (APC/C) [83]. Moreover, as demonstrated for eugenol [99], SEO components may also reduce the expression of genes encoding CDC20, further reinforcing APC/C inhibition. As a result, cells remain arrested in metaphase until the defect is resolved. This mechanism may explain the observed increase in the metaphase index. This interpretation is also supported by microscopic observations of partially decondensed chromatids remaining closely associated during metaphase, suggesting that cohesin was likely not degraded due to separase inhibition by securing in the absence of active APC/C (Figure 20) [88].

3.4. Disruption of Mitotic Progression via Epigenetic Modifications Induced by SEO

In a subpopulation of mitotic cells, the elevated metaphase index was accompanied by characteristic chromosomal aberrations. Highly condensed prophase foci localized to telomeric regions of sister chromatids persisted into metaphase and acted as 'sticky ends' in anaphase, preventing full separation during telophase. This resulted in chromatin bridges that subsequently formed micronuclei. Such stickiness may arise from volatile compounds denaturing nuclear proteins and impairing segregation, or from disrupted chromatin folding and organization [28].

Alternatively, the SEO-induced increase in the metaphase index, and associated mitotic abnormalities may stem from impaired activity of PP1 and PP2A phosphatases (Figure 20). These enzymes act throughout the cell cycle, coordinating chromatin condensation, mitotic kinase activity, microtubule organization, and the metaphase–anaphase transition. While their suppression is required to initiate mitosis, timely reactivation is essential for successful progression through later stages, particularly metaphase and anaphase [111,112]. The hypothesis that SEO inhibits PP1/2A is supported by results obtained with okadaic acid, a specific inhibitor of these phosphatases, which induces comparable mitotic defects in *V. faba* [112]. Further support comes from SEO-induced disruptions in conserved histone H3 phosphorylation marks (H3T3Ph, H3S10Ph) affecting both their levels and distribution.

Phosphorylation of H3T3 by Haspin kinase is essential for proper chromosome condensation and spindle assembly checkpoint function, as it recruits the chromosomal passenger complex (CPC) containing Aurora B. The CPC corrects kinetochore–microtubule attachment errors, ensures accurate chromosome segregation, and later contributes to spindle disassembly [83,84,88,113]. Aurora B also catalyzes phosphorylation of H3S10. While the regulation of Haspin in plants remains unclear, studies in other organisms suggest control by Polo-like kinases (Plk1, absent in plants), a feedback interaction with the CPC/Aurora B complex, and Haspin autophosphorylation [114,115], with inactivation mediated by PP1 [116]. Aurora B is likely activated by mitotic CDKs and inactivated by PP1 and PP2A, which stabilize kinetochore–microtubule attachments [117]. Thus, precise temporal and

spatial regulation of both kinases is critical for correct chromatin dynamics during mitosis (Figure 20) [118].

In this study, SEO markedly altered the phosphorylation patterns of H3T3 and H3S10, indicating disruption of epigenetic regulation of mitosis. H3T3Ph showed stronger and more widely distributed signals, persisting abnormally into anaphase and telophase. This likely reflects impaired PP1/PP2A activity, leading to sustained Haspin activation and insufficient H3T3 dephosphorylation [116]. In contrast, H3S10Ph intensity was reduced, which may reflect several factors: impaired recruitment of the CPC (containing Aurora B) to chromosomes (possibly secondary to prolonged H3T3 phosphorylation); alterations in chromatin architecture limiting Aurora B's access to the S10 residue; or indirect inhibition of Aurora B through SEO-induced reduction in CDK levels. Taken together, PP1 and PP2A act on key protein complexes responsible for histone modifications, including the regulation of H3T3 and H3S10 phosphorylation. These modifications are essential for the proper recruitment and activity of the CPC (with Aurora B kinase), which in turn regulates microtubule–kinetochore attachments and spindle checkpoint signaling. Thus, disturbances in histone phosphorylation may impair CPC function, leading to excessive stabilization of microtubules, spindle malfunction, and mitotic delay (Figure 20).

3.5. Toxicological Risks Associated with β -Asarone

Essential oils are inherently prone to degradation through oxidation, iso- or polymerization, and dehydrogenation. Due to these properties, they are not considered highly toxic, with acute effects observed only at doses above 2 g/kg (oral or dermal), and their residues on plants remain minimal [15]. Despite the promising phytotoxic activity of SEO, its application as a bioherbicide requires consideration of β -asarone, the main constituent. Mammalian studies have documented its genotoxic and carcinogenic potential, particularly after metabolic activation to β -asarone epoxides, with liver damage and cardiac atrophy observed in rats; the oral LD₅₀ is 1010 mg/kg [35]. β - and α -asarone also show toxicity against key agricultural insect pests [119]. Importantly, concentrations of SEO effective for inhibiting plant growth (IC₅₀ is 0.05–0.3 g/L) are far below mammalian toxic doses, suggesting that selective application could minimize risks to humans and animals. Nevertheless, field use should account for β -asarone toxicological limits, and further studies are needed on its degradation and environmental residue persistence.

4. Materials and Methods

4.1. Plant Material

Seeds of faba bean (*Vicia faba* L. subsp. *minor*, cv. Bobas; Danko, HR Sp. z o. o. Choryń, Poland), yellow lupine (*Lupinus luteus* L., cv. Baryt; PHR Sp. z o. o. Tulce, Poland), rapeseed (*Brassica napus* L. cv. Markus; HR Strzelce Sp. z o. o. Strzelce, Poland) and thale cress (*Arabidopsis thaliana* Col-0 (L.) Heynh; NASC, Nottingham, UK) were germinated on a filter paper in deionized water for 2–3 days at 23 °C in darkness, except *A. thaliana*, which, due to etiolation sensitivity, germinated in long-day conditions (16/8 h). Seedlings with equal-sized primary roots (about 2 cm *V. faba*, *L. luteus*, or 1 cm *B. napus* and 0.5 cm *A. thaliana*) were selected for further experiments.

4.2. Essential Oil Treatment

Sweet flag (*A. calamus*) essential oil (SEO) purchased from Etja, Poland, of known composition [97] was emulsified in a 1:4 (v/v) emulsifier-to-oil ratio by vigorous shaking for 15 min with a mixture of C14-18 and unsaturated C16-18—mono- and di-ethoxylated glycerides and ethoxylated *B. napus* oil. Seedlings were incubated in water-dissolved emulsified SEO, and IC₅₀ concentration (the concentration that inhibits root length increment

by 50%) was determined for each species. IC₅₀ concentration of SEO was: 0.03%—*V. faba*, 0.025%—*L. luteus*, 0.01%—*B. napus*, 0.005%—*A. thaliana*, respectively. Seedlings were incubated on filter paper in tightly sealed Parafilm Petri dishes in darkness for 24 h. The control was seedlings incubated in water and dissolved emulsifier in the concentration used for SEO emulsification. Ten root meristems were examined per species (Control series, emulsifier series—E, and SEO series—O), and four experimental repeats were performed.

4.3. DNA Staining for Mitotic and Mitotic Phase Indices

1 cm long apical root fragments were fixed in Carnoy's solution (99.8% ethanol: glacial acetic acid, 3:1 v/v) for 1 h, rinsed in absolute ethanol (3 times), and stored in 70% ethanol. Prior to DNA staining, root tips were rehydrated and hydrolyzed in 4 M HCl (for 2 h *V. faba*; 1 h *L. luteus*, *B. napus*; 30 min *A. thaliana*) and stained in Feulgen reaction using Schiff's reagent (pararosaniline; Sigma-Aldrich, St. Louis, MI, USA) for 1 h [120]. Cut-off meristems were squashed in a drop of 45% acetic acid onto microscopic slides and frozen by placing them on the surface of a dry ice cube. Then, cover slides were removed and the preparations were rinsed in 70% ethanol, air-dried, and mounted in Canada balsam.

4.4. Western Blot

Proteins were extracted from the root meristematic parts using Pierce Plant Total Protein Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich). Their concentration was spectrophotometrically measured using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). 45 µg of proteins from each extract were separated on NuPAGE Novex 4–12% Bis-Tris gel (Thermo Fisher Scientific) and immediately transferred onto polyvinylidene fluoride (PVDF) membrane, 0.2 µm pore size (Thermo Fisher Scientific). Membrane blocking, antibody incubation, and chromogenic detection were performed using WesternBreeze Chromogenic Kit, anti-rabbit (Thermo Fisher Scientific) according to the manufacturer's protocol. Cdc2 proteins were detected using polyclonal Anti-CDC2 antibodies (Agriserä, Vännäs, Sweden) diluted 1:3000, which cross-react with the conserved plant homolog CDKA; therefore, the term 'Cdc2' is used throughout this study. Actin was detected using monoclonal actin antibodies (Sigma-Aldrich) diluted 1:4000. The chromogenic reaction was run for 60 and 30 min, respectively. PVDF membranes were visualized using ProXima 2750 imaging platform (Isogen Life Sciences, De Meern, The Netherlands). Microdensitometric analysis of the PVDF membrane was performed using GelAnalyzer 23.1.1 (available at www.gelanalyzer.com (accessed on 1 March 2025) by Istvan Lazar Jr., PhD, and Istvan Lazar Sr., PhD. After loading the membrane image, baselines and bands were automatically detected by the program. Peak intensities were measured by adjusting the band visible on the lane profile and subtracting it from the baseline.

4.5. Immunocytochemical Staining of β -Tubulin and Actin

Excised, apical parts of roots were fixed in 4% (m/V) paraformaldehyde dissolved in MTSB buffer (50 mM PIPES, 5 mM EGTA, 5 mM MgSO₄, pH 7.0) for 45 min (4 °C) and washed three times with PBS (Sigma-Aldrich). Then root tips were macerated with a citrate-buffered mixture of 2.5% pectinase, 2.5% pectolyase, and 2.5% cellulase (Sigma-Aldrich), at pH 5.0 and 37 °C for 30 min (*V. faba* and *L. luteus*) or 20 min (*B. napus*) or 15 min (*A. thaliana*). After the digestion solution was removed, root tips were washed with PBS as before and squashed onto Super Frost Plus microscope slides (Menzel-Gläser, Thermo Fisher Scientific, Waltham, MA, USA) in a drop of PBS. Following freezing with dry ice, coverslips were removed, and air-dried cells on the slides were permeabilized with PBS-buffered 0.5% Triton X-100 (Sigma-Aldrich) for 15 min, pretreated with blocking buffer (5% BSA, 0.5% Triton X-100 dissolved in PBS) for 50 min (room temperature), briefly rinsed in PBS

and incubated with mouse monoclonal anti- β tubulin primary antibodies (Sigma-Aldrich) dissolved in 1:750 ratio in PBS containing 1% w/v BSA and 0.3% v/v Triton X-100; antibody buffer and in monoclonal actin antibodies (Sigma-Aldrich). Following overnight incubation in darkness and a humidified atmosphere at 4 °C, slides were rinsed three times in PBS and incubated at 23 °C for 90 min with secondary goat anti-mouse antibodies conjugated with Alexa Fluor 488 dissolved in a 1:500 ratio in antibody buffer. After that, the slides were rinsed three times in PBS and stained with 15 μ M 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 15 min. After another rinsing in PBS, the slides were embedded in PBS: glycerol mixture (9:1) with 2.3% DABCO (2.3% diazabicyclo[2.2.2]octane; Sigma-Aldrich).

4.6. Immunocytochemical Detection of Phosphorylated H3S10 and H3T3 Histones

Apical fragments of *V. faba* and *B. napus* roots were fixed in 4% (m/V) paraformaldehyde dissolved in PBS buffer for 45 min at 4 °C and washed three times with PBS. Tissue maceration and slide preparation were described above. For H3S10Ph detection, slides were preincubated for 50 min in PBS-buffered 8% BSA and 0.1% Triton X-100 at 23 °C and rinsed twice with PBS buffer for 5 min each. Then, slides were incubated with anti-H3S10Ph antibodies dissolved at 1:300 in PBS containing 1% BSA overnight at 4 °C in darkness in a humidified atmosphere.

To detect H3T3Ph, slides were incubated in permeabilizing solution (0.5% Triton X-100 in PBS) for 15 min at 23 °C, then briefly rinsed and incubated in blocking buffer containing 5% BSA and 0.3% Triton X-100 dissolved in PBS for 50 min at 23 °C. Slides were briefly rinsed and incubated overnight with anti-H3T3Ph antibodies (Cell Signaling Technology, Danvers, MA, USA) dissolved at 1:400 in PBS containing 1% BSA and 0.3% Triton-X100 at 4 °C in darkness in a humidified atmosphere.

After the incubation with primary antibodies stopped, rinsing, incubation in secondary antibodies solution, DAPI staining, and slide mounting procedures were described above.

4.7. Microscopic Observations, Measurements, and Analyses

Mitotic indexes and mitotic phase indexes were calculated with the use of Nikon Eclipse E100 microscope (Nikon, Tokyo, Japan) by analyzing 5000 cells from 5 preparations stained with Schiff's reagent. Calculations were made according to the formula: Mitotic index, MI = (number of dividing cells total number of cells⁻¹) · 100%; Phase index, PI = number of cells in a particular phase of mitosis · total number of cells in mitosis⁻¹) · 100%

Observations of fluorescence preparations were made using Nikon Eclipse E600W fluorescence microscope (Nikon, Tokyo, Japan) equipped with U2 filter (UVB light; λ = 340–380 nm) for DAPI, B2 filter (blue light; λ = 465–496 nm) for Alexa Fluor[®] 488, and G2 filter (green light; λ = 540/25 nm) equipped with a Nikon DS-Fil CCD camera (Nikon, Tokyo, Japan) or Zeiss AxioImager. A1 fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with an AxioCam ERc5s CCD camera (Carl Zeiss, Jena, Germany). All images were recorded at the same exposure time. Quantitative analyses of fluorescence intensity were made in ImageJ software ver. 1.54p [121] after converting color images into greyscale and expressed in arbitrary units.

4.8. Statistical Analyses

Statistical analyses and outcomes visualization were performed in GraphPad Prism 10 software. Differences between groups were evaluated using Student's *t*-test or the Mann–Whitney test. Statistical significance, denoted by asterisks (*) or hash symbols (#), was attributed to *p*-values ≤ 0.05 .

5. Conclusions

The results indicate that SEO reduces the proportion of mitotically active cells and disrupts mitotic progression in representatives of both Fabaceae (*V. faba*, *L. luteus*) and Brassicaceae (*B. napus*, *A. thaliana*). Application of species-specific IC₅₀ concentrations revealed the universality of SEO's main effects while also demonstrating species-specific differences in response intensity.

The action of SEO proved to be multifaceted, encompassing molecular mechanisms of cell cycle regulation, dynamic remodeling of cytoskeletal elements (microtubules and actin filaments), and mitosis-specific histone modifications such as H3T3 and H3S10 phosphorylation. These epigenetic alterations exhibited interspecific differences in both intensity and spatial distribution.

The reduction in the number of mitotic cells results from both replication stress and S-phase prolongation, caused by oxidative DNA damage, as confirmed in earlier studies, and from a decrease in the level of the key cell cycle regulatory kinase Cdc2 (a protein that normally remains relatively stable throughout the cell cycle), as demonstrated in *V. faba* and *B. napus* by Western blotting.

Mitotic disturbances were also associated with excessive stabilization of microtubules and actin filaments, and with restricted dynamic reorganization of the spindle and phragmoplast, observed in all studied species by immunofluorescence as stronger lateral interactions and persistent stabilization at each mitotic stage. It is likely that microtubule-associated proteins (MAPs) mediate these lateral interactions; under reduced Cdc2 levels, they may fail to undergo phosphorylation required for weakening their microtubule binding and enabling proper separation.

Mitotic defects were further linked to altered timing and intensity of H3T3 phosphorylation, which is essential for correcting erroneous kinetochore–microtubule attachments and for proper spindle organization, as shown in *V. faba* and *B. napus* by immunofluorescence. This disruption is likely due to SEO interference with PP1/2A phosphatases, responsible both for H3 dephosphorylation and for Haspin kinase inactivation, although this assumption requires further validation.

Disturbances in H3T3 phosphorylation timing may also have hindered CPC/Aurora B recruitment to chromatin, thereby weakening H3S10 phosphorylation, as demonstrated in *V. faba* and *B. napus*. In addition, the observed decrease in Cdc2 levels could further reduce Aurora B activity, limiting its ability to phosphorylate H3S10 and to ensure proper chromatin condensation into metaphase chromosomes and accurate sister chromatid separation. This mechanism, however, requires further confirmation.

From the perspective of the potential use of SEO as a bioherbicide, it is particularly important to emphasize its capacity to target multiple components of the cell cycle. This multi-targeted mode of action may reduce the risk of plants rapidly developing resistance mechanisms, which is a key advantage of such compounds in agricultural practice.

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Abbreviations

The following abbreviations are used in this manuscript:

APC/C	Anaphase-promoting complex/cyclosome
ATM	Ataxia-telangiectasia mutated kinase
ATR	Ataxia-telangiectasia and Rad3-related protein
BMF3	BUB1/MAD3 family protein 3
CAK	Cdk-activating kinase
Cdc2	Cell division control 2
CDC20	Cell division cycle 20 protein
CDC25	Cell division cycle 25 phosphatase
CDKs	Cyclin-dependent kinases
CKIs	Cyclin-dependent kinase inhibitors
CPC	Chromosomal passenger complex
Cyc	Cyclin
DP	Dimerization partner
MAD2	Mitotic arrest deficient 2
MAPs	Microtubule-associated proteins
MAPK	Mitogen-activated protein kinase
MCC	Mitotic checkpoint complex
MPF	Mitosis-promoting factor
NACK1	NPK1-activating kinesin-like protein 1
PP1-2A, B, C	Protein Phosphatase 1-2A, B, C
PPB	Preprophase band
Rb	Retinoblastoma protein
RBR	Retinoblastoma-related proteins
ROS	Reactive oxygen species
SAC	Spindle assembly checkpoint
SMC	Structural maintenance of chromosome protein
TPX2	Targeting Protein of XKLP2
γ -TuRC	γ -tubulin ring complex

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Original Article

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Ultrastructural, signaling, and epigenetic responses to *Acorus calamus* L. essential oil in root meristems of Fabaceae and Brassicaceae seedlings

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Keywords: allelopathy, allelochemical, phytotoxicity, hexokinase, MAPK, epigenetics, cell ultrastructure

Significance statement: This study explores the largely unknown epigenetic and signaling alterations underlying the phytotoxic effects of essential oils, using sweet flag essential oil as a model. The findings provide new insights into plant responses to allelopathic stress and lay the groundwork for further research and the development of innovative bioherbicides.

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40 **Summary**

41 Essential oils are promising agents for sustainable crop protection. Here, we investigated
42 the impact of emulsified sweet flag (*Acorus calamus*) essential oil (SEO) on root meristem
43 cells of Fabaceae (*Vicia faba*, *Lupinus luteus*) and Brassicaceae (*Brassica napus*,
44 *Arabidopsis thaliana*). Cytological, ultrastructural, immunocytochemical, and qPCR
45 analyses revealed profound alterations in organelle integrity, metabolism, and chromatin
46 organization. SEO inhibited hexokinase activity while damaging plastid and
47 mitochondrial membranes, thereby transmitting stress signals to the nucleus, increasing
48 hexokinase, sucrose synthase, and MAPK kinase transcripts, and enhancing MAPK
49 (p44/42) phosphorylation. Chromatin remodeling was marked by global condensation
50 and transcriptional repression, as shown by increased DNA methylation, gain of
51 H3K4Me2, loss of permissive marks (H3T45Ph, H4K5Ac), reduced 5-ethynyluridine
52 incorporation, and nucleolar vacuolization. Despite repression, stress-related
53 transcription persisted: H3K9Ac indicated activation of stress-responsive genes, while
54 focal H4K5Ac suggested selective maintenance of rRNA synthesis. Redistribution of
55 H3K56Ac from a uniform pattern to larger foci reflected DNA repair responses, consistent
56 with SEO-induced DNA damage. Comparative analysis showed greater chromatin
57 plasticity and repair in *V. faba*, whereas *B. napus* exhibited stronger repression and
58 replication stress. Collectively, SEO induced a multilayered stress response integrating
59 metabolic disruption, chromatin remodeling, and transcriptional reprogramming, with
60 species-specific outcomes relevant to sustainable weed management.

1. Introduction

Replacing synthetic herbicides with products of plant secondary metabolism, as an environmentally friendly alternative, remains a major challenge due to the complexity of the problem and the slow progress of research aimed at elucidating the mechanisms of plant phytotoxicity (Kostina-Bednarz *et al.*, 2023; Schandry and Becker, 2020; Verdeguer *et al.*, 2020; Wróblewski, Piotrowska-Niczyporuk, *et al.*, 2025). Essential oils (EOs), one of the most abundant groups of such metabolites, are considered a promising alternative to synthetic herbicides. To date, more than 3000 essential oils have been identified, each typically comprising 20 - 60 active compounds (Raveau *et al.*, 2020; Verdeguer *et al.*, 2020). In addition to their phytotoxic potential, relevant for weed eradication in crop production, EOs can also be used for pest control and may even act as biostimulators of plant growth (Hara, 2020; Pavela and Benelli, 2016). Depending on their application as bioherbicides or plant growth-promoting agents, EOs induce specific forms of stress in plants, differing in both mechanism and intensity (Kostina-Bednarz *et al.*, 2023).

While the physiological aspects of EO-induced stress, such as altered antioxidant activity, modulation of hormone signaling, and stress-related protein synthesis, are already partially understood, the pathways of signal perception and transduction leading to regulatory responses at the chromatin level remain largely unexplored. In particular, little is known about how dynamic epigenetic modifications of chromatin orchestrate the precise activation or repression of specific genomic regions, thereby finely modulating the synthesis of certain components while concurrently downregulating others during EO stress (Schandry and Becker, 2020; Verdeguer *et al.*, 2020; Assadpour *et al.*, 2024; Foncoux *et al.*, 2023; Gupta *et al.*, 2023; Islam *et al.*, 2024; Kostina-Bednarz *et al.*, 2023; Álvarez-Rodríguez *et al.*, 2022). These challenges are further compounded by species-specific differences in plant responses, likely rooted in the diversity of their genomes and epigenomes, which shape their adaptive capacities (Gao *et al.*, 2018).

The sessile nature of plants has driven the evolution of diverse adaptive strategies, enabling them to maintain homeostasis and sustain growth through highly developed stress-sensing capabilities (Gallusci *et al.*, 2023). Among the plant's primary sensory structures is the root, which not only ensures water and nutrient uptake but also acts as a communication hub between the plant and the soil environment, thereby influencing

the plant's fate under stress (Ramachandran *et al.*, 2024; Ryan *et al.*, 2016). From germination and primary root emergence onward, further root development originates in the meristematic tissue located in the apical zone of the root. Due to the intensive proliferation of meristematic cells, this zone is one of the most sensitive structures in the plant. To ensure proper DNA distribution during cell division, it must detect and integrate stress signals, enabling the inhibition of cell proliferation when necessary (Shimotohno *et al.*, 2021). The root apical meristem has also been proposed as a component of the broader meristematic connectome, encompassing all primary and secondary apical meristems, which function as an interconnected structure coordinating the plant's adaptation to environmental conditions throughout its lifespan and integrating bidirectional signaling between root and shoot (Chiatante *et al.*, 2021).

Sophisticated signaling networks in plants integrate a wide range of pathways, including those mediated by reactive oxygen species (ROS), sugars, plant hormones, and protein-based signaling cascades such as mitogen-activated protein kinases (MAPKs), along with many other interconnected events. Together, these systems fine-tune plant adaptation processes by modulating chromatin structure and gene expression patterns to enhance stress tolerance (Mansoor *et al.*, 2023).

Sugars act not only as primary energy sources but also as important signaling molecules. In this context, sucrose synthase (SuSy; EC 2.4.1.13) is a pivotal enzyme that catalyzes the reversible cleavage of sucrose into UDP-glucose and fructose, thereby providing precursors for both primary metabolism and cell wall biosynthesis (Wenqi, 2025), whereas invertase (INV; EC 3.2.1.26) catalyzes the irreversible hydrolysis of sucrose into glucose and fructose (Ferne *et al.*, 2002). Together, the activities of SuSy and INV generate hexose substrates for hexokinase (HXK; 2.7.1.1), one of the most evolutionarily conserved sugar sensors. HXK catalyzes the phosphorylation of hexoses into glucose-6-phosphate and fructose-6-phosphate, thereby linking sugar metabolism to central cellular energy pathways. In addition to its metabolic role, HXK is also directly involved in sugar sensing, although the precise mechanisms underlying this signaling function remain to be fully elucidated (Jeandet *et al.*, 2022; Li and Zhao, 2024; Wu *et al.*, 2023). In plant cells, hexokinases (HXKs) are found not only in the cytosol but also associated with the outer membranes of mitochondria and plastids, as well as within chloroplast stroma

and the Golgi apparatus (J.,-I., Cho *et al.*, 2006; Damari-Weissler *et al.*, 2007; Damari-Weissler *et al.*, 2006; da-Silva *et al.*, 2001; Giese *et al.*, 2005; Olsson *et al.*, 2003; Wiese *et al.*, 1999). Mitochondria-bound HXKs can interact with the actin cytoskeleton, which is necessary for certain HXK-dependent glucose signaling events (Balasubramanian *et al.*, 2007). Moreover, AtHxk1 has been detected in the nuclear fraction, implying a potential role in direct transcriptional regulation (Y.,-H., Cho *et al.*, 2006; Claeysen and Rivoal, 2007). Acting in concert with other key regulatory proteins, such as SNF-related protein kinase 1 (SnRK1) and the target of rapamycin (TOR) kinase, HXK participates in the regulation of plant growth and in orchestrating responses to a variety of abiotic and biotic stresses (Li and Zhao, 2024). The functional characterization of HXKs is complicated by the presence of other sugar-phosphorylating enzymes, such as glucokinases and fructokinases, which may also contribute to cellular signaling pathways (da-Silva *et al.*, 2001; Oesterhelt and Gross, 2002; Pego and Smeekens, 2000). Thus, HXK functions at the interface of sugar metabolism and signaling, linking carbohydrate status to stress-responsive pathways such as mitogen-activated protein kinase (MAPK) cascades.

MAPKs, which are localized in the cytoplasm and nucleus (and in some cases associated with membranes), are widely recognized as central mediators of stress signaling. The MAPK signaling cascade is a highly conserved pathway among eukaryotes and consists of three hierarchically organized classes of kinases: MAP kinase kinase kinases (MAPKKKs), MAP kinase kinases (MAPKKs), and MAP kinases (MAPKs). Upon stress perception, these kinases are sequentially activated through phosphorylation, resulting in the amplification and transmission of the stress signal. Activation of the MAPK cascade has been documented under a wide range of adverse conditions, including cold, salinity, and heavy metal exposure (Liang *et al.*, 2022; Mansoor *et al.*, 2023). Signal transduction leading to the activation of stress-responsive genes involves chromatin remodeling. Dynamic chromatin remodeling involves the concurrent relaxation and condensation of protein-coding and regulatory regions, enabling precise control of gene expression (Chen *et al.*, 2022; Ramos-Cruz *et al.*, 2021). This regulatory process is mediated by chemical modifications of DNA, post-translational modifications (PTMs) of histones, and the action of small RNAs (Ramos-Cruz *et al.*, 2021).

DNA methylation plays a protective role in maintaining genome integrity by transcriptionally silencing transposable elements and other potentially harmful sequences (Sriraman *et al.*, 2020). Beyond this defensive function, it also participates in the physiological regulation of fundamental processes in plants, including tissue differentiation, organismal development, and adaptation to a wide range of environmental stresses encountered throughout the plant life cycle (Miryeganeh, 2021; Shafiq and Khan, 2015; Zhang *et al.*, 2010). DNA methylation is generally regarded as a repressive epigenetic mark. It is maintained by DNA methyltransferases and by *de novo* 5-cytosine methylation in the RNA-directed DNA methylation pathway (RdDM). Increased levels of DNA methylation, which enhance chromatin condensation and silence gene expression, are frequently associated with higher plant susceptibility to stress (Gupta and Salgotra, 2022; Ramos-Cruz *et al.*, 2021). This repression can be reversed by the action of DNA demethylases (Lu *et al.*, 2023), which remove methyl groups and promote chromatin relaxation. When this relaxation is accompanied by the accumulation of specific histone marks, it may signal the rapid activation of defense-related genes, thereby enhancing the plant's adaptive potential under stress (Ramos-Cruz *et al.*, 2021).

In addition to DNA methylation, histones undergo a wide range of epigenetic modifications that act in a coordinated manner to form the so-called 'histone code', which is interpreted by regulatory proteins and shapes the chromatin state. PTMs of histones typically occur at the N-terminal tails protruding from the nucleosome core. Among the various modifications, differing in the type of chemical group added, lysine acetylation is of particular importance in stress responses. This modification is catalyzed by histone acetyltransferases (HATs), whereas its removal is mediated by histone deacetylases (HDACs). HAT activity generally promotes chromatin relaxation and transcriptional activation, while HDAC activity favors chromatin condensation and transcriptional repression (Ramos-Cruz *et al.*, 2021). Moreover, MAPKs can modulate histone acetylation indirectly, for example, by phosphorylating HDACs, thereby influencing stress-responsive gene expression during biotic stress (Latrasse *et al.*, 2017). Although HATs and HDACs exert opposing effects, they act in a coordinated manner to finely tune gene expression under adverse environmental conditions (Ramos-Cruz *et al.*, 2021). During the acetylation process, an acetyl group, donated by Acetyl-CoA, is added

to the ϵ -amine group of lysine residues in the histone N-terminal tails (Hu *et al.*, 2019). Acetyl-CoA is a metabolic intermediate generated in mitochondria, plastids, or peroxisomes through metabolic pathways such as the tricarboxylic acid (TCA) cycle, fatty acids β -oxidation, or glycolysis, the latter being initiated by hexokinase activity (Hu *et al.*, 2019; Wu *et al.*, 2023).

Like DNA, histones can also undergo methylation or demethylation, mediated by histone methyltransferases and histone demethylases - enzymes often referred to as 'writers' and 'erasers' of epigenetic marks. The functional outcome of histone methylation depends on both the specific amino acid residue and the methylation state (mono-, di-, or trimethylation), making it a versatile regulatory signal rather than a universally repressive one (Gupta and Salgotra, 2022; Xu and Jiang, 2020). For instance, in rice, H3K4Me3 is positively correlated with active gene transcription (Hu *et al.*, 2020), whereas H3K4Me2 is regarded as a repressive epigenetic mark (Liu *et al.*, 2019). The dynamic balance between HMT and HDM activities allows cells to rapidly adjust gene expression in response to environmental challenges (Chen *et al.*, 2022).

Histones can also undergo phosphorylation, which typically occurs on serine, threonine, or tyrosine residues within histone tails. This modification is catalyzed by protein kinases - such as MAPKs, Aurora kinases, and cyclin-dependent kinases (CDKs) - while phosphatases serve as the corresponding 'erasers' removing phosphate groups (Nowak and Corces, 2004; Rossetto *et al.*, 2012). Histone phosphorylation plays a central role in dynamic chromatin remodeling, contributing to transcriptional regulation, DNA repair, programmed cell death, and cell cycle progression. In plants, MAPK cascades have been shown to indirectly phosphorylate histones, thereby linking stress signaling to chromatin-based control of gene expression (Rayapuram *et al.*, 2018). As such, histone phosphorylation represents a highly reversible and versatile epigenetic mechanism that couples environmental cues with chromatin organization and genome stability.

In the search for essential oils with potential applications in broad plant protection strategies, we focused on sweet flag (*Acorus calamus* L.) essential oil (SEO) due to its distinctive phytochemical profile, particularly the abundance of phenylpropanoids such as asarones (Satyal *et al.*, 2013; Szczeblewski *et al.*, 2022), whose specific molecular effects in plant cells are still not fully understood. Although SEO has a long history of use

in traditional Chinese medicine and its biological activity has been documented in animal models and *in vitro* systems (Lam *et al.*, 2017; Rajamanikkam *et al.*, 2019; Saki *et al.*, 2020). Knowledge about its action in plants remains limited. In addition to its intriguing chemical properties, practical factors also favored its use, including the ready availability of plant material and the considerable biomass of rhizomes, which represent an efficient source of oil. This choice allowed us to establish a model system to investigate how a naturally derived stressor can influence root growth, cell cycle, signaling pathways, and epigenetic regulation, thereby linking fundamental cellular responses to processes of potential interest in agricultural research.

To evaluate SEO impact, we selected two legume species from the Fabaceae family (*Vicia faba* L. var. *minor* and *Lupinus luteus* L.), both of which are major forage crops with large seeds but are highly susceptible to weed competition during early developmental stages. Their vulnerability is further exacerbated by the restricted number of selective herbicides available for legumes (European Food Safety Authority (EFSA) *et al.*, 2020; Pawłowski, 2018). For comparison, two Brassicaceae species were included as representatives of competing plants (*Brassica napus* L. and *Arabidopsis thaliana* L.). Although *B. napus* is cultivated as an oilseed crop, its volunteer seedlings often emerge in subsequent rotations and interfere with legume production (Weber *et al.*, 2014). In contrast, *A. thaliana* functions both as a widespread weed and as a model organism, making it a valuable subject for future mechanistic and molecular investigations.

Our previous research focused on the effects of SEO at IC₅₀ concentrations, precisely adjusted for each species (0.03% for *V. faba*, 0.025% for *L. luteus*, 0.01% for *B. napus*, and 0.005% for *A. thaliana*) to ensure comparable stress intensity. We investigated its impact on the growth and cytophysiological responses of seedling primary roots, with particular emphasis on the closely associated proliferative activity in their apical region, including DNA replication during the S phase and its subsequent segregation into daughter cells during mitosis, in order to determine whether the responses are species- or family-specific, or rather individual. Overall, the results revealed that SEO acted primarily through the induction of oxidative stress and, while the underlying disturbances in cell cycle progression were mediated by similar mechanisms across all studied species, Fabaceae primary roots displayed greater tolerance to SEO exposure, whereas

Brassicaceae were more susceptible and exhibited distinct biochemical responses (Wróblewski, Krajewski, *et al.*, 2025; Wróblewski, Piotrowska-Niczyporuk, *et al.*, 2025).

In this study, we aimed to investigate whether SEO modulates the activity of the root apical meristem through sugar- and MAPK-dependent signaling pathways that ultimately lead to characteristic epigenetic reprogramming. We hypothesized that the SEO-induced reduction in root growth under stress is associated with perturbations in metabolic signaling as well as with the activation of MAPK cascades, which reconfigure the epigenetic state of chromatin to safeguard genome stability. This process is further accompanied by the selective activation of chromatin regions that sustain transcriptional programs essential for cell survival, while overall transcriptional activity is reduced to a minimum.

Thus, we analyzed HXK activity, and the transcript levels of SuSy, HXK, and MAPK kinase (MKK), alongside phosphorylated MAPK accumulation, global DNA methylation, and histone modifications (H3K4Me₂, H3T45Ph, H4K5Ac, H3K9Ac, H3K56Ac) associated with chromatin reorganization, including condensation and relaxation, which contribute to both gene repression and activation, as well as to S-phase progression and the DNA damage response. To assess downstream effects of epigenetic reprogramming, we measured transcriptional activity by labeling nascent RNA with the ethynyl uridine (EU) assay, evaluated nucleolar morphology using AgNOR staining, and examined ultrastructural alterations through transmission electron microscopy.

2. Results

In the present study, most experiments were focused on two representative species, *V. faba* (Fabaceae) and *B. napus* (Brassicaceae), rather than on all four species tested in the previous assays. This choice was guided by findings showing that members of both families respond to SEO through similar regulatory mechanisms, with the main differences being in the intensity rather than the nature of the response. Therefore, *V. faba* and *B. napus* were selected as model representatives of the two families, allowing for comprehensive and technically feasible cytological or immunofluorescence analyses of signaling pathways and epigenetic modifications.

2.1. Assessment of hexokinase activity in relation to mitochondrial and plastid ultrastructure and related gene expression

Hexokinase (HXK) activity in root meristem cells of *V. faba* and *B. napus* was visualized through the accumulation of the enzymatic reaction product, precipitated nitroblue formazan, which appeared as purplish-dark blue granules predominantly surrounding the nuclei (Figure 1). This localization pattern suggests HXK activity within specific organelles, most likely mitochondria and/or plastids (Figure 1B, D), consistently with their localization identified in our earlier studies (Polit, 2009; Polit and Ciereszko, 2009). In addition to organelle-associated staining, diffuse coloration was also observed throughout the cytoplasm, resulting in a uniform bluish-purple hue across the entire cell. Therefore, staining intensity was evaluated separately in the organelle-associated granules (Figure 1E, F) and in the surrounding cytoplasmic areas (Figure 1G, H). Negative control incubations, in which the immediate enzymatic substrates (glucose or NAD⁺) were omitted, showed no detectable formazan staining, confirming the specificity of the reaction (Figure 1A, C).

Analysis of staining intensity in cells exposed to the emulsifier revealed generally stronger organelle-associated labeling in *V. faba* (Figure 1E) and enhanced cytoplasmic staining in both *V. faba* and *B. napus* compared to the control (Figure 1G, H), as reflected by the higher median values (180 % and 110 % of control in *V. faba* and *B. napus*, respectively). As shown by violin plots, the subpopulation of cells with weak cytoplasmic staining was

either absent or markedly reduced. At the same time, no cells exhibited higher staining levels than those observed in the control.

Following 24-hour exposure to the IC₅₀ concentration of SEO, a marked reduction in formazan staining was observed in the organelle-associated regions of meristematic cells in both species compared with emulsifier-treated seedlings (Figure 1E, F). When considering the broader cytoplasmic staining surrounding organelles, SEO treatment caused a significant decrease in staining intensity in *V. faba* relative to the emulsifier-treated group, while in *B. napus*, the reduction was significant compared with both control and emulsifier treatments (Figure 1G, H). Given the organelle-associated localization of HXK activity, mitochondrial and plastid ultrastructure was subsequently assessed to determine whether structural alterations could accompany the observed enzymatic patterns.

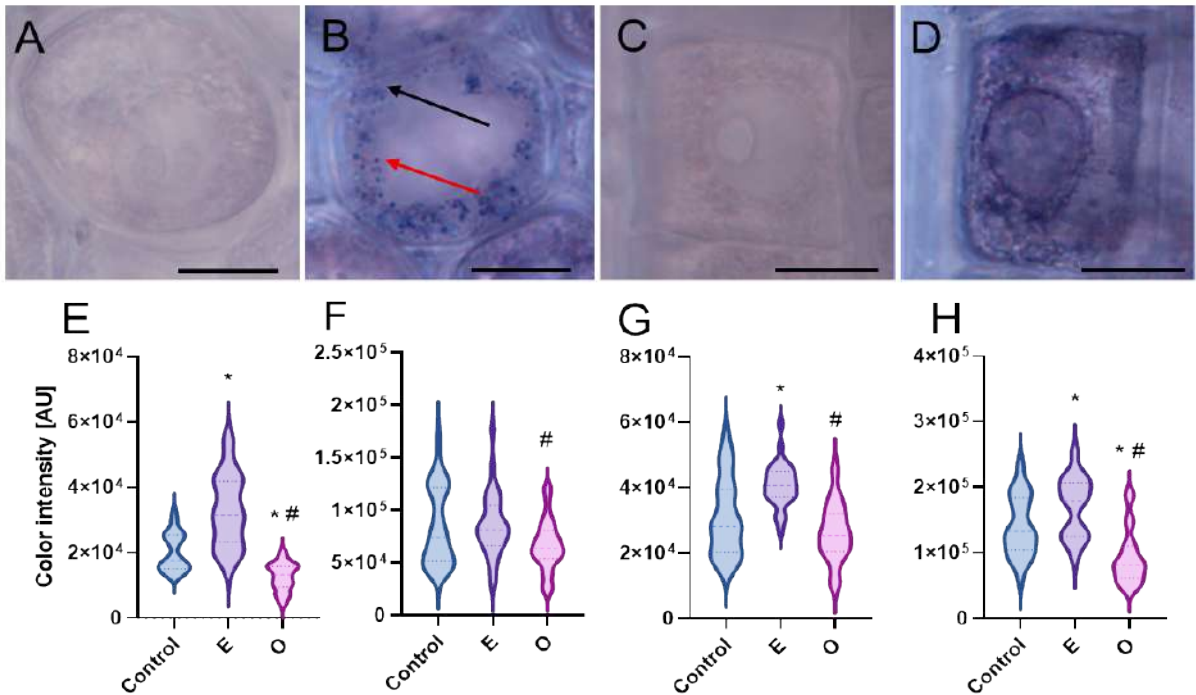


Figure 1. Histochemical detection of hexokinase (HXK) activity in root meristem cells. *V. faba* root meristem cells in transverse (A, B) and longitudinal (C, D) sections. Negative controls (A, C) were prepared by omitting glucose or NAD. In contrast, sections incubated with the complete reaction mixture (B, D) show the reduction of nitroblue tetrazolium (NBT) to insoluble blue formazan, reflecting HXK activity. Black arrow indicate stained foci corresponding to mitochondria and/or plastids, whereas red arrow indicate cytoplasmic regions analyzed for staining intensity. Scale bar: 10 μ m. Quantitative analysis of staining intensity associated with organelles (E, F) and cytoplasm (G, H) in *V. faba* (E, G) and *B. napus* (F, H) was performed after 24 h seedling incubation in water – Control, emulsifier solution – E, or emulsified SEO at the IC₅₀ concentration – O. The width of each violin plot represents the distribution of values (AU, arbitrary units), with medians indicated by dashed lines, and quartiles by dotted lines. Statistical differences were assessed for $n = 30$ in each series using the Mann–Whitney test at $p \leq 0.05$. Asterisk (*) and hash (#) marks denote significant differences compared to the control and the emulsifier, respectively.

In the ultrastructural images of control and emulsifier-treated root meristem cells, mitochondria and plastids exhibited typical morphology (Figure 2A, C). These organelles, round or oval, were surrounded by a distinct double membrane, with visible regions corresponding to translocase complexes, where the intermembrane space appeared locally narrowed and electron dense. Well-developed mitochondrial cristae filled the inner space, while plastids frequently contained light starch grains of variable size. Both types of organelles were regularly positioned around the cell nucleus. In contrast, cells exposed to SEO displayed pronounced alterations in organelle organization (Figure 2B, D). Mitochondria often underwent fusion, with their matrix showing extensive electron-lucent regions instead of densely packed cristae. Discontinuities of the outer mitochondrial membrane were also occasionally observed. Plastids, in turn, frequently appeared depleted of starch reserves, contained prolamellar bodies, and adopted elongated, irregular shapes, with signs of disorganization of the outer plastid envelope.

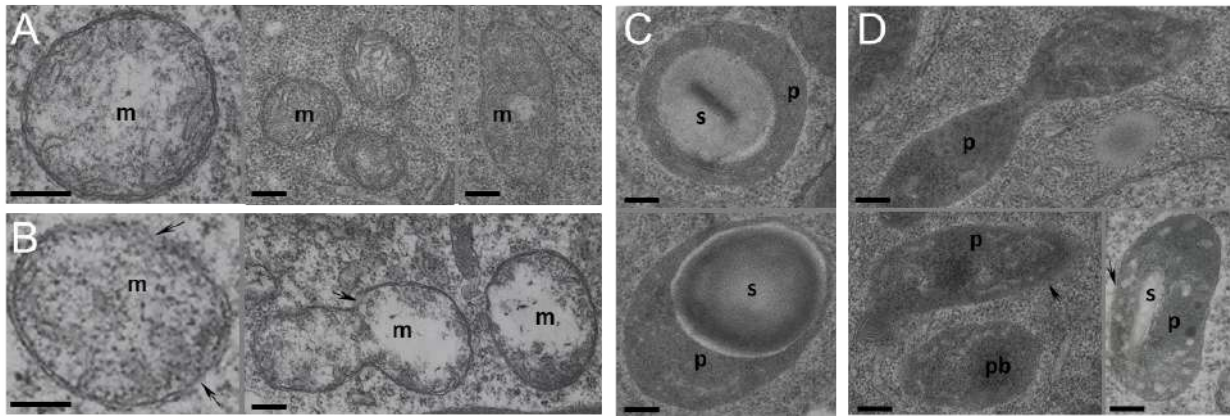


Figure 2. Mitochondria and plastid ultrastructure in root meristem cells of *V. faba* and *B. napus* after 24 h of seedling incubation in water – Control (A, C), or emulsified SEO at the IC_{50} concentration (B, D); m – mitochondrion, p – plastid, s – starch, pb – prolamellar body, arrowheads indicate damage to the membrane surrounding the organelles. Scale bar: 200 nm.

To complement the assessment of enzymatic activity and organelle ultrastructure, transcript levels of HXK and sucrose synthase (SuSy) were evaluated by qPCR (Figure 3). SuSy, catalyzing the reversible cleavage of sucrose into UDP-glucose and fructose, was included because it can provide hexose substrates for HXK-dependent pathways and UDP-glucose for stress-related processes. Even exposure to the emulsifier alone altered transcript levels compared with untreated controls, with HXK showing a modest decrease and SuSy approximately doubling relative to control. Following SEO treatment, HXK transcripts exhibited a slight increase relative to control, whereas SuSy expression

rose substantially, reaching approximately 3.5-fold of control levels. The observed differences between HXK and SuSy transcript levels highlight the prominent role of SuSy in maintaining carbohydrate flux and signaling capacity in meristematic cells under stress conditions.

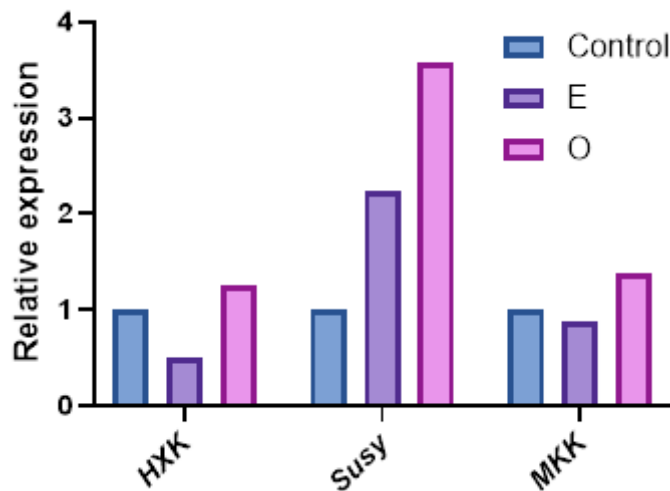


Figure 3. Gene expression changes in *V. faba* primary roots after 24 h of seedling incubation in water - Control, emulsifier solution - E or emulsified SEO at the IC₅₀ concentration - O. The data are presented as the mean of four technical replicates.

2.2. Detection of MAPK phosphorylation and upstream MKK transcription

To determine the localization and activity levels of MAPKs, we performed immunofluorescent labeling in *V. faba* (Figure 4A-D) and Western blot analysis in *B. napus* (Figure 4E, F), using polyclonal anti-phospho-p44/42 MAPK (Erk1/2) antibodies specific to the dually phosphorylated TEY motif (Winnicki *et al.*, 2015). These antibodies detect the active forms of plant MAPKs, most notably MPK3 and MPK6, which are functional homologs of mammalian Erk1/2. Immunolabeling in *V. faba* combined with fluorescence intensity analysis revealed that phosphorylated MAPKs localize to the nuclei as sharply defined, punctate nuclear foci (Figure 4A-C and D). In both the control group (Figure 4A-A'') and seedlings incubated with the emulsifier alone (Figure 4B-B''), the MAPK signals appeared as small foci. In contrast, treatment with the emulsified SEO resulted in a pronounced increase in signal intensity (Figure 4C-C''), with median fluorescence intensity doubling compared to both control and emulsifier-only treatments (Figure 4D).

In *B. napus*, immunolabeling did not reveal any distinct, punctate fluorescence signals in the nuclei. Therefore, phosphorylated MAPK levels were assessed via Western blotting (Figure 4E) followed by densitometric quantification (Figure 4F). In root meristems of control seedlings, a weak signal was observed for p44 MAPK (MPK3), while p42 MAPK (MPK6) remained barely detectable. Treatment with the emulsifier alone caused a slight increase in p44 MAPK levels, with no significant effect on p42. However, exposure to the IC₅₀ concentration of SEO for 24 h resulted in markedly elevated signals for both isoforms: p44 MAPK increased to 135% of the control (111% of emulsifier-only), and p42 MAPK rose to 221% relative to both control and emulsifier-treated seedlings.

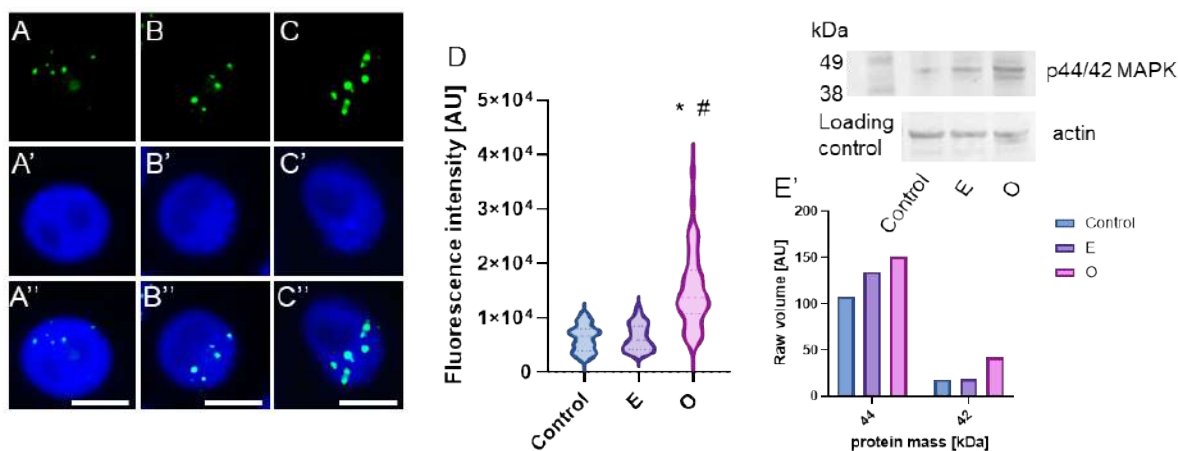


Figure 4. Immunofluorescence detection of phospho-p44/42 MAPK (Thr202/Tyr204; known as MPK3 and MPK6 plant homolog of mammalian Erk1/2) in root meristem cells of *V. faba* (A–D) and immunoblot detection of this phospho-protein in protein extracts from root meristem cells of *B. napus* (E, F) after 24 h seedling incubation in water – Control, emulsifier solution – E, or emulsified SEO at the IC₅₀ concentration – O. Nuclei counterstained with DAPI (A'–C'); merged images (A''–C''). Scale bar: 10 μ m. Quantitative analysis of fluorescence intensity (D): violin plots show the distribution of values (AU, arbitrary units), with medians indicated by dashed lines and quartiles by dotted lines. Statistical differences were assessed for $n = 50$ in each series using the Mann–Whitney test at $p \leq 0.05$. Asterisks (*) and hashes (#) denote significant differences compared with the control and emulsifier, respectively. Immunoblot (E) with actin (49–38 kDa) as loading control. Relative protein abundance between treatments determined by microdensitometric analysis of band intensity using Gel Analyzer software (F).

Given that MAPK activity is triggered through upstream kinases in the MAPK cascade, transcript levels of MAPK kinase (MKK), the immediate activator of MAPKs, were evaluated in *V. faba* by qPCR to determine whether transcriptional regulation contributes to the observed phosphorylation patterns (Figure 3). MKK was analyzed only in *V. faba* due to the availability of a suitable gene sequence for primer design; amplification in *B. napus* was not feasible. In seedlings treated with the emulsifier alone, MKK transcript levels remained comparable to the control. Exposure to the IC₅₀ concentration of SEO, however, resulted in a moderate increase, with MKK expression rising by approximately

one-third relative to control, consistent with its role as an upstream activator facilitating MAPK phosphorylation under stress conditions.

2.3. Epigenetic landscape

Since SEO treatment resulted in reduced HXK activity, increased MAPK activity, and alterations in transcript levels of HXK, SuSy, and MKK, the subsequent analyses focused on whether these metabolic and signaling changes were accompanied by modifications in the epigenetic landscape, including DNA methylation and histone marks.

2.3.1. Detection of 5-Methylcytosine (5-mC) and ultrastructural chromatin alterations

Immunolabeling with anti-5-mC antibodies revealed a nuclear immunopositive signal in root meristem cells of *V. faba* (Figure 5A–C) and *B. napus* (Figure 5D–F). The fluorescence intensity, reflecting the level of DNA methylation associated with transcriptional gene silencing, varied between experimental treatments in both species (Figure 5G–H). In *V. faba*, both control and emulsifier-treated nuclei exhibited low fluorescence intensity, with a further decrease observed in the emulsifier-treated group relative to the control (Figure 5G), suggesting a slight reduction in DNA methylation levels. In *B. napus*, fluorescence levels remained comparable between the control and emulsifier-treated seedlings (Figure 5H), indicating stable and relatively low baseline DNA methylation.

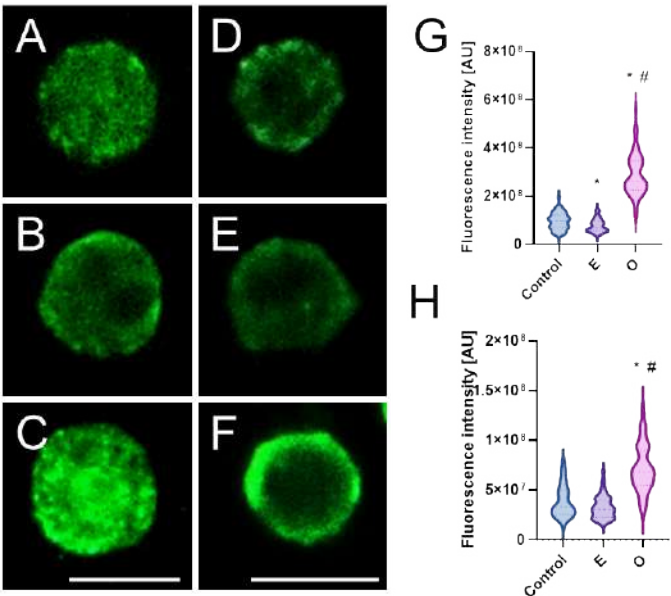


Figure 5. Immunofluorescence detection of 5-methylcytosine in root meristem cells of *V. faba* (A–C, G) and *B. napus* (D–F, H) after 24 h seedling incubation in water – Control (A, D), emulsifier solution – E (B, E), or emulsified SEO at the

IC₅₀ concentration – O (C, F). Scale bar: 10 μ m. Quantitative analysis of fluorescence intensity (G, H). Violin plots represent the distribution of values (AU, arbitrary units), with medians indicated by dashed lines and quartiles by dotted lines. Statistical significance was determined for $n = 60$ in each series using the Mann–Whitney test at $p \leq 0.05$. Asterisks (*) and hashes (#) denote significant differences compared with the control and emulsifier, respectively.

Exposure to the IC₅₀ concentration of emulsified SEO led to a marked increase in fluorescence intensity in both species (Figure 5C, F, G, H). In *V. faba*, the signal reached 271% of the control and 388% of the emulsifier median. Similarly, in *B. napus*, fluorescence levels increased to approximately 220% of both control and emulsifier-treated samples, indicating a strong SEO-induced hypermethylation effect.

The observed increase in DNA methylation was accompanied by alterations in the chromatin landscape, which were clearly discernible in TEM images of nuclei (Figure 6). The analyzed plant species differ in their nuclear DNA content, which underlies the occurrence of two distinct types of nuclei. *V. faba* cells, containing considerably more DNA than *L. luteus*, *B. napus*, and *A. thaliana*, are characterized by reticulate nuclei with distinct chromocenters. In control conditions, the ultrastructure of these nuclei displayed a mosaic arrangement of dispersed euchromatin and condensed heterochromatin (Figure 6A). By contrast, the latter three species possess euchromocentric nuclei, in which small aggregates of condensed chromatin are embedded within a background of diffuse euchromatin (Figure 6B). The nuclear envelope was clearly defined, consisting of two membranes separated by a narrow electron-lucent intermembrane space. Nuclear pore complexes were regularly distributed, appearing as electron-dense constrictions within the intermembrane space or as ring-like profiles on the nuclear surface, depending on the plane of section (Figure 6C).

Following treatment with SEO at IC₅₀ concentrations specific for each species, alterations in nuclear ultrastructure were evident (Figure 6D-F). Both in the nuclei of *V. faba* and the nuclei of the other species, the relative proportions of dispersed and condensed chromatin shifted toward an increased prevalence of heterochromatin (Figure 6D, E). Moreover, the nuclear envelope exhibited reduced regularity, with locally

widened intermembrane spaces and markedly less frequent nuclear pore complexes (Figure 6F).

Considering the observed increase in DNA methylation and the enhanced proportion of condensed chromatin, we next examined changes in histone modifications involved in fundamental nuclear processes, including DNA replication, repair, and transcription.

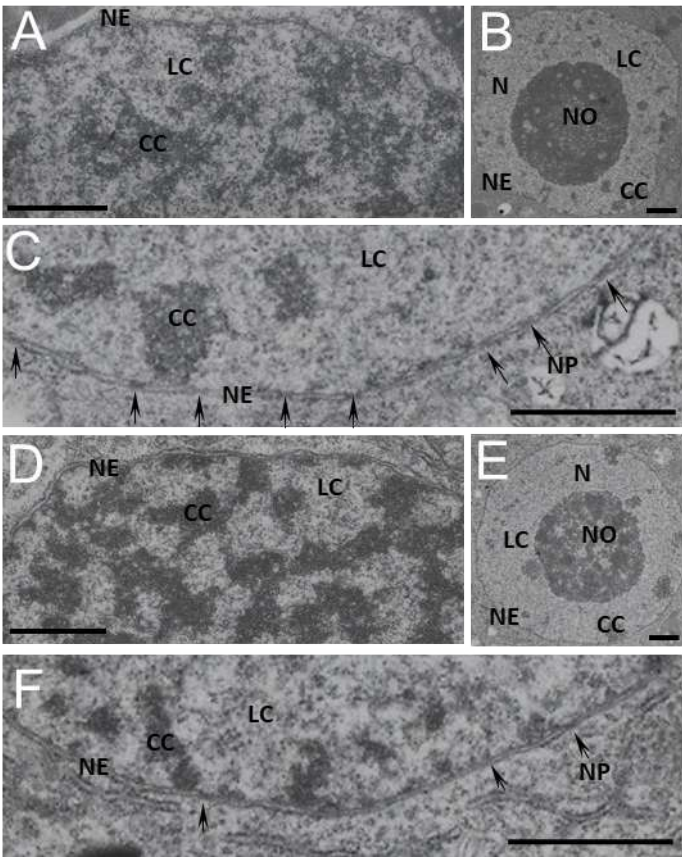


Figure 6. Ultrastructure of the nuclei in root meristem cells of *V. faba* (A, D) and *B. napus* (B, C, E, F) after 24 h of seedling incubation in water – Control (A, B, C), or emulsified SEO at the IC_{50} concentration (D, E, F); CC – condensed chromatin, LC – loose chromatin, N – nucleus, NE – nuclear envelope, NO – nucleolus, NP – nuclear pores. Scale bar: 1 μ m.

2.3.2. Dimethylation of H3K4

Immunofluorescence detection of the H3K4Me2 histone mark, which is frequently associated with gene promoter regions, was performed in root meristem cells of *V. faba* (Figure 7A–C) and *B. napus* (Figure 7D–F) using anti-H3K4Me2 antibodies. The signal pattern differed slightly between species; in *V. faba* cells, it appeared more granular, whereas in *B. napus* it was more homogeneous. Quantitative analyses of fluorescence intensity (Figure 7G, H) revealed comparable signal levels between the control and emulsifier-treated series in *V. faba*, while in *B. napus*, the emulsifier caused a statistically

significant decrease in H3K4Me2 levels, with the median fluorescence intensity dropping to approximately 70% of the control.

In both species, treatment with IC₅₀ SEO resulted in a pronounced increase in H3K4Me2 accumulation (Figure 7C-C'' and F-F''). In *V. faba*, the fluorescence intensity reached approximately 140% of the median values recorded in both the control and emulsifier-treated groups (compare Figure 7A-A'', B-B'', G). Similarly, in *B. napus*, the signal increased by about 120% relative to the control and by approximately 170% in comparison to the emulsifier-treated samples (compare Figure 7D-D'', E-E'', H), indicating a clear SEO-induced enhancement of this epigenetic mark.

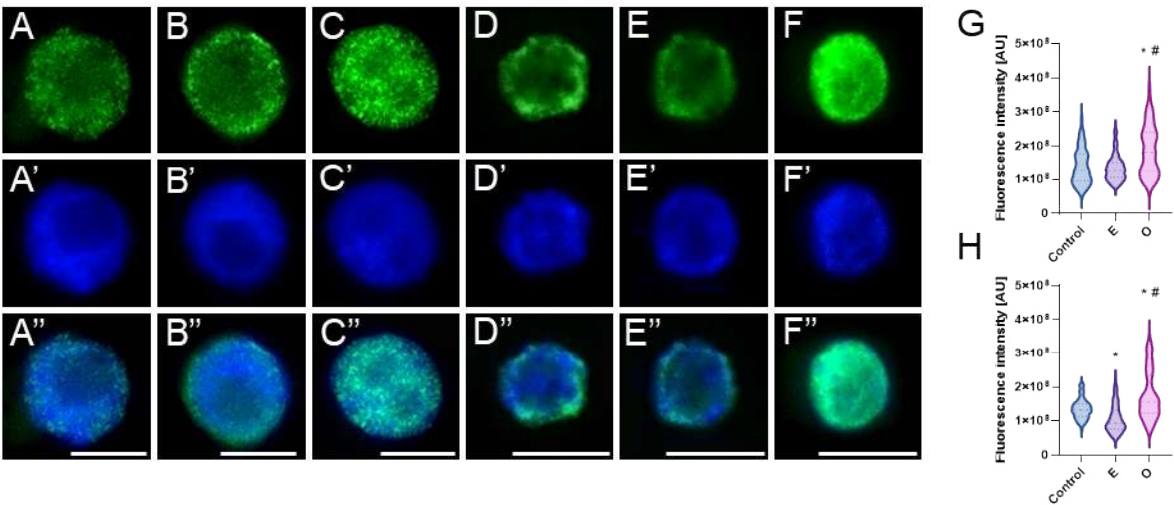


Figure 7. Immunofluorescence detection of the H3K4Me2 in root meristem cells of *V. faba* (A–C, G) and *B. napus* (D–F, H) after 24 h seedling incubation in water – Control (A, D), emulsifier solution – E (B, E), or emulsified SEO at the IC₅₀ concentration – O (C, F). Nuclei counterstained with DAPI (A'–F'); merged images (A''–F''). Scale bar: 10 μm. Quantitative analysis of fluorescence intensity (G, H). Violin plots represent the distribution of values (AU, arbitrary units), with medians indicated by dashed lines and quartiles by dotted lines. Statistical significance was determined for n = 120 in each series using the Mann–Whitney test at p ≤ 0.05. Asterisks (*) and hashes (#) denote significant differences compared with the control and emulsifier, respectively.

2.3.3. Phosphorylation of H3T45

Phosphorylation of histone H3 at threonine 45 (H3T45Ph), which is often associated with loosening of DNA–histone interactions during fundamental DNA-templated processes, was assessed in root meristematic cells of *V. faba* (Figure 8A–C) and *B. napus* (Figure 8D–F) using immunocytochemical detection with anti-H3T45Ph antibodies. In both species, the nuclear chromatin exhibited a similar labelling pattern, characterized by a fine-grained, homogeneously distributed signal, with a tendency to form delicate foci of increased intensity in the peripheral regions of the nuclei. Both microscopic observations

and quantitative fluorescence analyses indicated a high basal level of H3T45Ph within the nuclear chromatin in control plants of both species (Figure 8A-A", D-D", G, H). Treatment with the emulsifier led to a reduction in H3T45Ph accumulation, with median fluorescence intensity values decreasing to approximately 75% of the control in *V. faba* and to 72% in *B. napus* (Figure 8B-B", E-E", G, H). Exposure to IC₅₀ SEO caused a further marked decline in this histone modification: in *V. faba*, the median fluorescence intensity dropped to 66% of the control and 85% of the emulsifier-treated group, while in *B. napus*, the values reached 40% and 56%, respectively (Figure 8C-C", F-F", G, H).

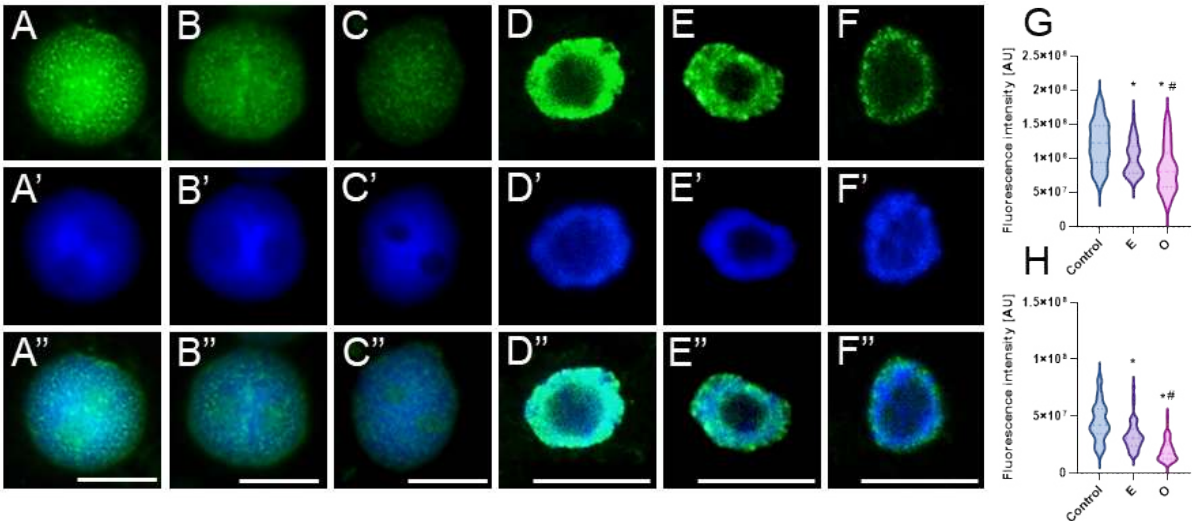


Figure 8. Immunofluorescence detection of the H3T45Ph in root meristem cells of *V. faba* (A–C, G) and *B. napus* (D–F, H) after 24 h seedling incubation in water – Control (A, D), emulsifier solution – E (B, E), or emulsified SEO at the IC₅₀ concentration – O (C, F). Nuclei counterstained with DAPI (A'–F'); merged images (A''–F''). Scale bar: 10 μm. Quantitative analysis of fluorescence intensity (G, H). Violin plots represent the distribution of values (AU, arbitrary units), with medians indicated by dashed lines and quartiles by dotted lines. Statistical significance was determined for $n = 50$ in each series using the Mann–Whitney test at $p \leq 0.05$. Asterisks (*) and hashes (#) denote significant differences compared with the control and emulsifier, respectively.

2.3.4. Acetylation of H4K5

Immunodetection of H4K5Ac, present in both eu- and heterochromatic regions of interphase nuclei during chromatin relaxation associated with replication and transcription, was performed in root meristem cells of *V. faba* and *B. napus* (Figure 9). Analysis of control cells revealed that the intensity and distribution of the signal differed slightly between species, which may be attributed, among other factors, to differences in total DNA content and the organization of heterochromatic regions (Figure 9A–A", B–B", C–C" and H–H", I–I", J–J"). Despite these differences, two principal patterns of H4K5Ac labeling were identified in interphase nuclei of both species: (i) a diffuse euchromatic

pattern, with homogeneous labeling throughout the chromatin (Figure 9A-A", H-H"), and (ii) distinct immunopositive clusters superimposed on the diffuse signal, corresponding to condensed chromatin regions – heterochromatic pattern (Figure 9B-B", I-I"). Both homogeneous and clustered patterns exhibited variable fluorescence intensity, ranging from weak to very strong. In *V. faba*, an additional nucleolar-specific labeling pattern was detected in less than 1% of meristematic nuclei. In these few nuclei, which otherwise showed homogeneous interphase labeling, the signal appeared as distinct fibrous structures within the nucleolus or along its periphery. (Figure 9G-G"). This pattern was occasionally observed in nuclei exhibiting clustered labeling and, in all mitotic figures, localized to the nucleolus organizer region (NOR) of nucleolus-forming chromosomes (Figure 9C-C"). The NOR-associated signal was detectable from prophase through late anaphase, standing out clearly against the dark chromosome arms lacking fluorescence. In contrast, *B. napus* cells did not display a characteristic nucleolar labeling pattern in interphase nuclei (which may have been below the resolution limit of the microscope due to the lower DNA content), but mitotic chromosomes primarily showed a relatively homogeneous acetylation signal with brighter foci appearing against this background (Figure 9J-J"). The emulsifier did not induce changes in the morphology of fluorescent signals and did not significantly affect the average fluorescence intensity (quantification was limited to cells with homogeneous signal distribution to ensure comparability between experimental series and avoid variability caused by differences in cluster number among nuclei at different cell cycle stages, Figure 9N, O). In contrast, treatment with IC₅₀ SEO resulted in a clear reduction of H4K5Ac accumulation in *V. faba* (Figure 9D-D", E-E", F-F") and *B. napus* (Figure 9K-K", L-L", M-M"), affecting homogeneous and clustered labeling patterns in interphase nuclei, as well as NOR-associated labeling in mitotic chromosomes of *V. faba* and *B. napus* or along entire chromosome arms in *B. napus*. Quantitative analysis of homogeneous labeling in *V. faba* (Figure 9N) showed that fluorescence levels dropped to approximately 65 % and 58 % of the median values recorded in control and emulsifier-treated samples, and in *B. napus* (Figure 9O), they fell to about 55 %, reflecting a pronounced suppressive effect. Notably, in *V. faba*, a distinctive difference compared with control and emulsifier-treated samples was the sharp increase in the proportion of interphase nuclei (reaching nearly 21 %) that

displayed fibrous H4K5Ac structures within the nucleolus and/or along its periphery (Figure 9G-G’’).

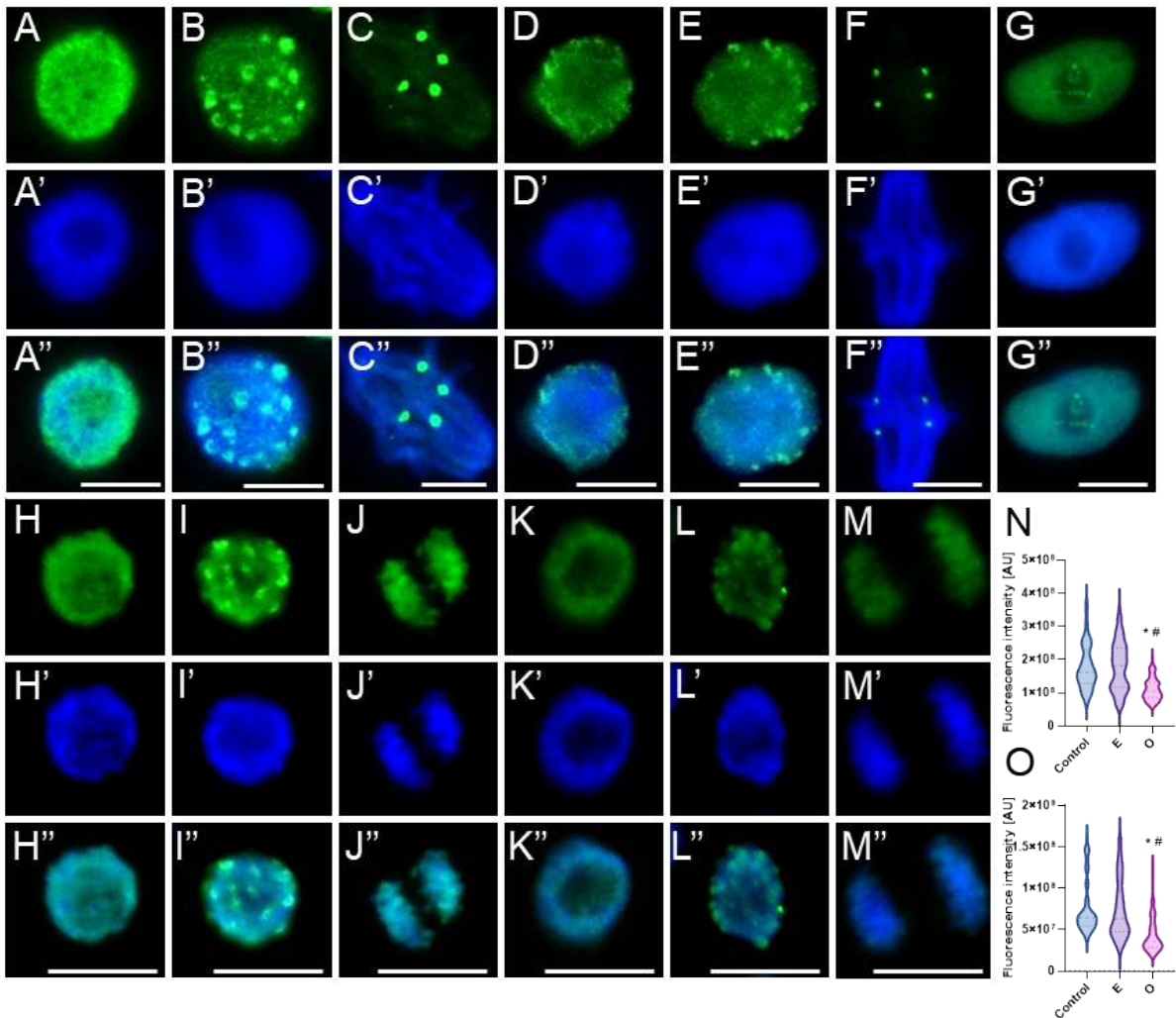


Figure 9. Immunofluorescence detection of the H4K5Ac in root meristem cells of *V. faba* (A–G, N) and *B. napus* (H–M, O) after 24 h seedling incubation in water – Control (A–C, H–J, N, O), emulsifier solution – E (N, O), or emulsified SEO at the IC₅₀ concentration – O (D–G, K–M, N, O). Nuclei counterstained with DAPI (A'–M'); merged images (A''–M''). Scale bar: 10 μ m. Quantitative analysis of fluorescence intensity (N, O). Violin plots represent the distribution of values (AU, arbitrary units), with medians indicated by dashed lines and quartiles by dotted lines. Statistical significance was determined for $n = 120$ in each series using the Mann–Whitney test at $p \leq 0.05$. Asterisks (*) and hashes (#) denote significant differences compared with the control and emulsifier, respectively.

2.3.5. Acetylation of H3K9

Histone H3K9 acetylation (H3K9Ac), which occurs in promoter regions of genes activated in response to stress, was evaluated in root meristem cells of *V. faba* (Figure 10A–C) and *B. napus* (Figure 10D–F) using immunocytochemical labeling with anti-H3K9Ac antibodies. Fluorescence intensity measurements revealed low acetylation levels in control samples (Figure 10A–A'', D–D'', G, H). Application of the emulsifier resulted in a

moderate increase in H3K9ac accumulation, reaching up to 150% of the control median in *V. faba* and 105% in *B. napus* (Figure 10B-B", E-E", G, H). The most substantial enhancement was observed after exposure to IC₅₀ SEO. In *V. faba*, median fluorescence rose to 160% of the control and 105% of the emulsifier group (Figure 10C-C", G). In *B. napus*, values approached 300% relative to both reference groups (Figure 10F-F", H), indicating a strong acetylation-promoting effect of SEO.

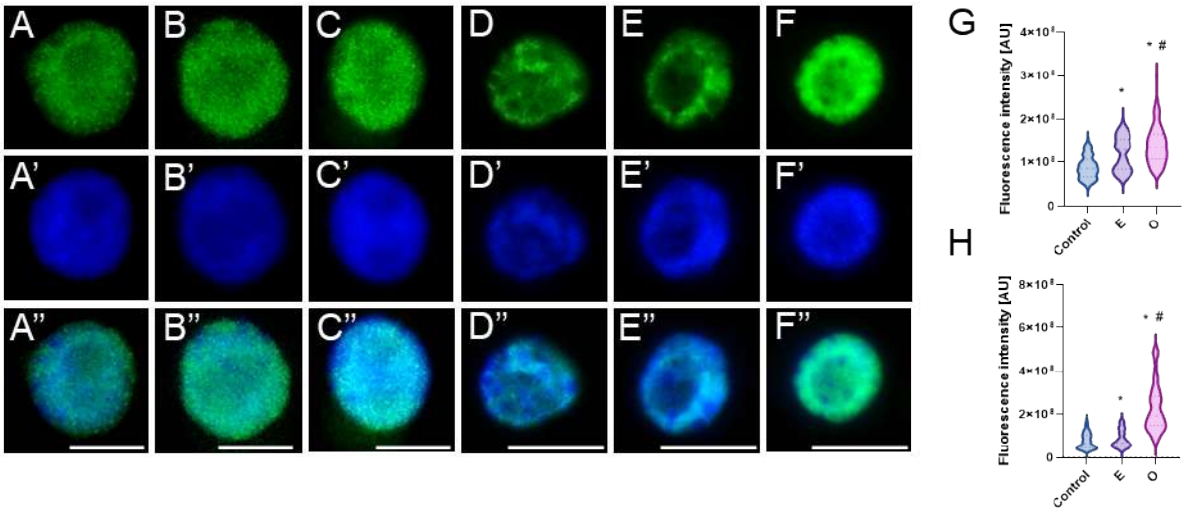


Figure 10. Immunofluorescence detection of the H3K9Ac in root meristem cells of *V. faba* (A–C, G) and *B. napus* (D–F, H) after 24 h seedling incubation in water – Control (A, D), emulsifier solution – E (B, E), or emulsified SEO at the IC₅₀ concentration – O (C, F). Nuclei counterstained with DAPI (A'–F'); merged images (A''–F''). Scale bar: 10 μ m. Quantitative analysis of fluorescence intensity (G, H). Violin plots represent the distribution of values (AU, arbitrary units), with medians indicated by dashed lines and quartiles by dotted lines. Statistical significance was determined for $n = 120$ in each series using the Mann–Whitney test at $p \leq 0.05$. Asterisks (*) and hashes (#) denote significant differences compared with the control and emulsifier, respectively.

2.3.6. Acetylation of H3K56

Immunocytochemical detection of H3K56Ac, a histone modification associated with sites of DNA damage, was performed in root meristematic cells of *V. faba* (Figure 11A–C) and *B. napus* (Figure 11D–F) using anti-H3K56Ac antibodies. In control and emulsifier-treated meristems, immunofluorescence was distributed across the entire chromatin in the form of fine granules, producing the impression of homogeneous nuclear labeling (Figure 11A-A", B-B", D-D", E-E"). In contrast, SEO-treated nuclei displayed distinct fluorescent foci within both chromatin and perinucleolar regions, which were absent in control samples. These foci were more numerous and appeared against a darker chromatin background in *V. faba* (Figure 11C-C") than in *B. napus* nuclei (Figure 11F-F"), indicating a reduction in the diffuse, homogeneous H3K56Ac signal in *V. faba*.

Quantitative analysis confirmed that global H3K56Ac levels in *V. faba* nuclei were comparable between control and emulsifier-treated cells (Figure 11G). However, after 24 h of treatment with SEO at IC₅₀ concentrations, diffuse nuclear fluorescence was markedly reduced, reaching approximately 75% of the values observed in control and emulsifier-treated samples. In *B. napus* cells, no significant differences in global H3K56Ac fluorescence intensity were detected, which is consistent with the lower frequency of foci and the overall retention of diffuse nuclear labeling (Figure 11H). The SEO-induced decrease in diffuse fluorescence together with the persistence of localized foci points to a shift from global acetylation toward focal H3K56 acetylation, suggesting a chromatin environment permissive for targeted DNA repair.

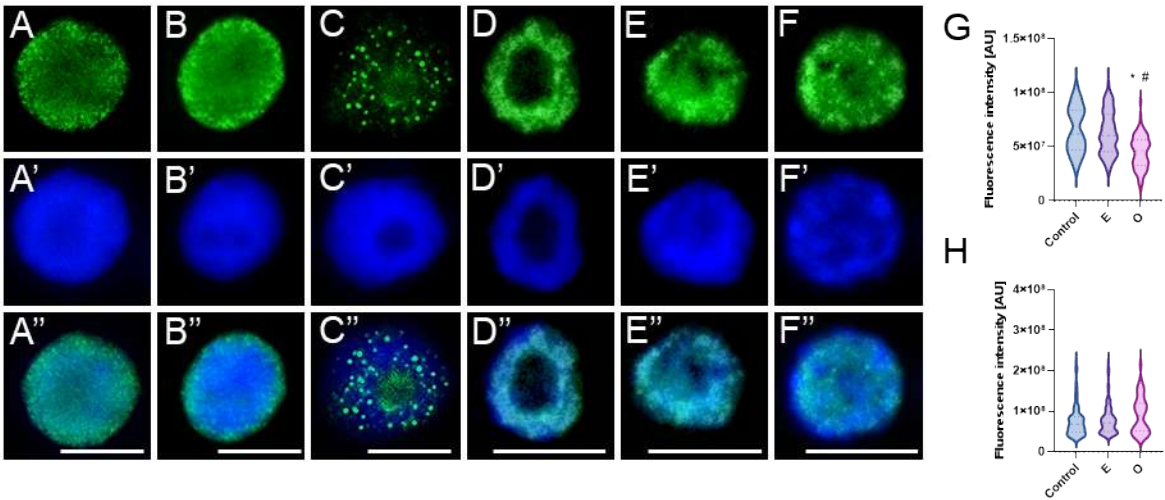


Figure 11. Immunofluorescence detection of the H3K56Ac in root meristem cells of *V. faba* (A–C, G) and *B. napus* (D–F, H) after 24 h seedling incubation in water – Control (A, D), emulsifier solution – E (B, E), or emulsified SEO at the IC₅₀ concentration – O (C, F). Nuclei counterstained with DAPI (A'–F'); merged images (A''–F''). Scale bar: 10 μ m. Quantitative analysis of fluorescence intensity (G, H). Violin plots represent the distribution of values (AU, arbitrary units), with medians indicated by dashed lines and quartiles by dotted lines. Statistical significance was determined for $n = 120$ in each series using the Mann–Whitney test at $p \leq 0.05$. Asterisks (*) and hashes (#) denote significant differences compared with the control and emulsifier, respectively.

2.4. Evaluation of transcriptional activity and nucleolar morphology

Having characterized the stress-induced changes in DNA and histone modifications, we next examined how these epigenetic adjustments correlate with transcriptional activity in root meristem cells. Transcriptional activity in root meristem cells of *V. faba* (Figure 12A–D, G–H), *L. luteus* (Figure 12I), *B. napus* (Figure 12E–F, J), and *A. thaliana* (Figure 12K) was assessed using the 5-ethynyluridine (EU) incorporation assay. This method employs an uridine analogue that, following incorporation into nascent RNA, can be visualized via

the Click-iT reaction and fluorescence microscopy. A weaker fluorescence signal was detected in the nucleoplasm, whereas the most prominent and homogeneous signals were localized within the nucleoli, reflecting active rRNA synthesis. Among the analyzed species, most possess two nucleolar organizer chromosomes (NORs), except for *B. napus*, which has four. However, due to the relatively small genome size in all species except *V. faba*, the nucleolar material in interphase nuclei is generally organized into a single, large, centrally positioned nucleolus, rather than multiple distinct nucleoli. In contrast, *V. faba*, characterized by a large genome and two NOR-bearing chromosomes, displays cell cycle-dependent variation in nucleolar morphology: cells may contain either two smaller, separate nucleoli (Figure 12A, C) or a single enlarged nucleolus formed by their fusion (Figure 12B, D). This morphological variability was considered in the quantitative analysis: fluorescence intensity and nucleolar area were measured separately for nuclei containing one or two nucleoli, while in the remaining species, measurements were taken from the single, large nucleolar area.

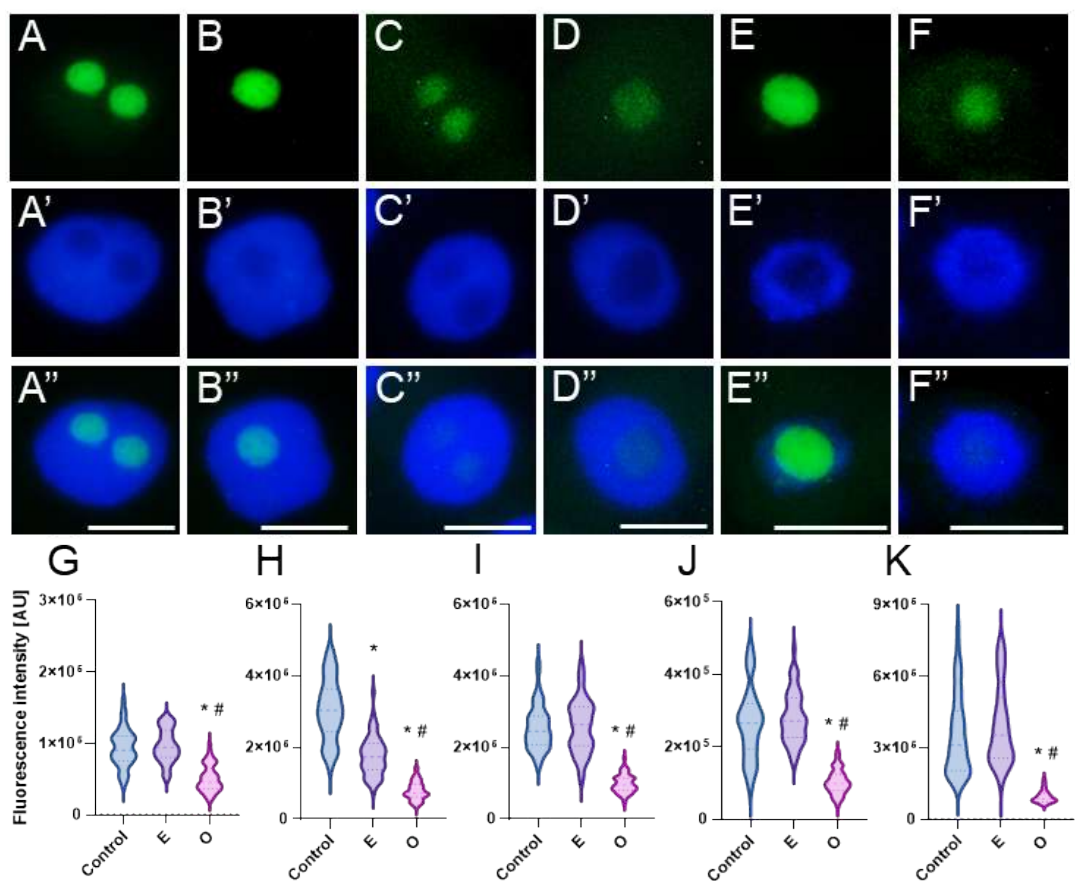


Figure 12. Transcription detected by the 5-ethynyluridine (EU) incorporation assay in root meristem cells of *V. faba* (A–D, G, H) containing either two smaller, separate nucleoli (A, C, G) or a single enlarged nucleolus formed by their fusion

(B, D, H) and in *B. napus* (E–F, I) as well as in *L. luteus* (J) and *A. thaliana* (K) after 24 h seedling incubation in water – Control (A, B, E), emulsifier solution – E, or emulsified SEO at the IC₅₀ concentration – O (C–D, F). Nuclei counterstained with DAPI (A'–F'); merged images (A''–F''). Scale bar: 10 μm. Quantitative analysis of fluorescence intensity (G–K). Violin plots represent the distribution of values (AU, arbitrary units), with medians indicated by dashed lines and quartiles by dotted lines. Statistical significance was determined for n = 50 in each series using the Mann–Whitney test at p ≤ 0.05. Asterisks (*) and hashes (#) denote significant differences compared with the control and emulsifier, respectively.

In the control samples, nucleoli exhibited intense and homogeneous fluorescence signals, reflecting robust rRNA transcriptional activity (Figure 12A–A'', B–B'', E–E''). Treatment with the emulsifier alone preserved this pattern, maintaining fluorescence intensity at levels comparable to the control (Figure 12G–K), with the only notable reduction observed in the fused nucleoli of *V. faba* (Figure 12H). In contrast, exposure to IC₅₀ SEO resulted in a pronounced decline in nuclear fluorescence intensity ranging from 50 - 25 % of control or emulsifier series (Figure 12C–C'', D–D'', F–F'') across all examined species (Figure 12G–K), indicating a substantial inhibition of transcriptional processes.

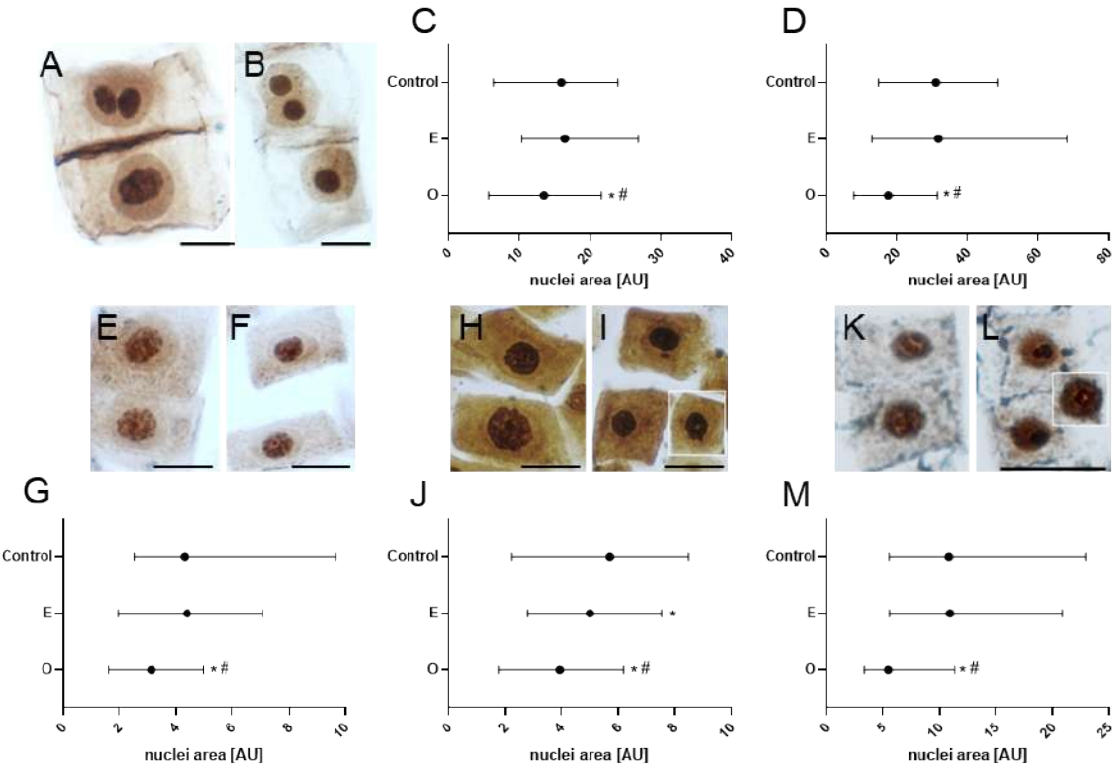
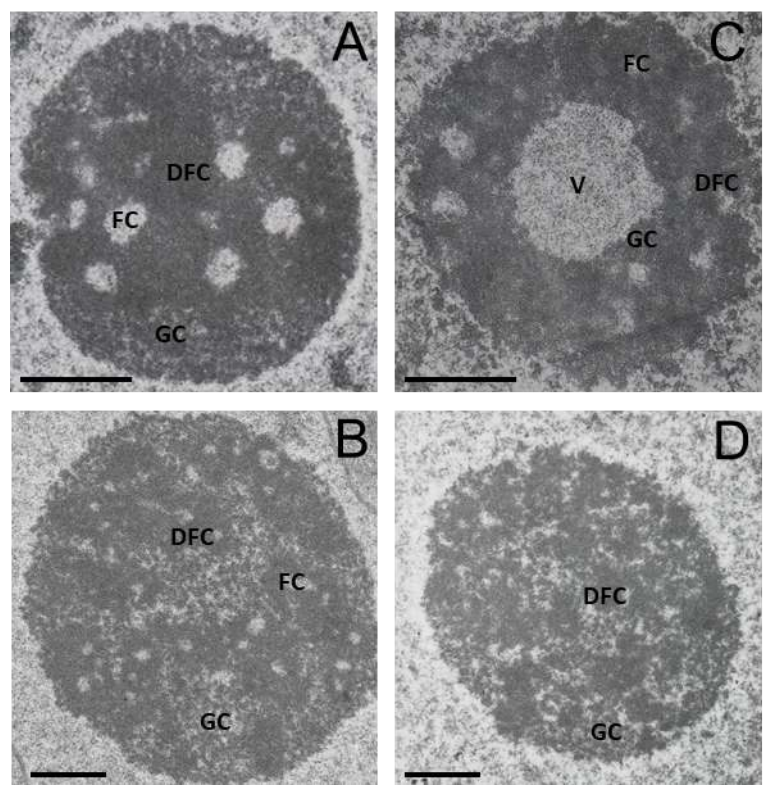


Figure 13. Ag-NOR staining of nucleoli and quantitative analysis of nucleoli area in root meristem cells of *V. faba* (A, B), containing either two smaller, separate nucleoli (E) or a single enlarged nucleolus formed by their fusion (D) and in *L. luteus* (E, F, G) as well as in *B. napus* (H, I, J) and *A. thaliana* (K, L, M) after 24 h seedling incubation in water – Control (A, E, H, K), emulsifier solution – E, or emulsified SEO at the IC₅₀ concentration – O (B, F, I, L). Scale bar: 10 μm. Lines represent the distribution of values (AU, arbitrary units), with medians indicated by dashed lines and quartiles by dotted lines. Statistical significance was determined for n = 50 in each series using the Mann–Whitney test at p ≤ 0.05. Asterisks (*) and hashes (#) denote significant differences compared with the control and emulsifier, respectively.

To complement the quantitative analyses, nucleolar morphology was further examined using Ag-NOR staining, which provided additional structural insights (Figure 13). Ag-NOR staining revealed distinct nucleolar patterns between control/emulsifier and SEO-treated cells. In control and emulsifier samples, nucleoli displayed a granular, heterogeneous staining pattern with alternating darker and lighter regions, consistent with active rDNA transcription sites (Figure 13A, E, H, K). In contrast, SEO treatment produced more uniformly stained nucleoli, indicative of altered nucleolar organization (Figure 13B, F, I, L). Occasionally, small oval light areas were observed within nucleoli, which may correspond to nucleolar vacuoles (the boxed area in Figure 13I, L). Analysis of nucleolar area revealed no significant alterations in response to the emulsifier compared with the control (Figure 13C, D, G, M), with the exception of a slight reduction observed in *B. napus* (Figure 13J). In contrast, treatment with IC₅₀ SEO caused a marked decrease in nucleolar size, varying across species and nucleolar types. In *V. faba*, the reduction reached approximately 15% in single nucleoli and nearly 40% in fused nucleoli (Figure 13A-D). Similarly, SEO treatment reduced nucleolar area by about 30% in *L. luteus* (Figure 13E-G), 30 % in *B. napus* (Figure 13H-J), and no more than 50% in *A. thaliana* (Figure 13K-M). These findings indicate that SEO not only suppressed transcriptional activity but also induced a substantial shrinkage of nucleolar structures.

Observations of Ag-NOR-stained nucleoli were corroborated and further detailed by ultrastructural analysis using TEM (Figure 14A-D). In control cells of all examined species, nucleoli displayed a typical tripartite organization, with clearly defined fibrillar centers (FC) surrounded by dense fibrillar component (DFC) regions containing rDNA transcription products. The DFC was further enclosed by an extensive granular component (GC) representing preribosomal subunits (Figure 14A, B). Occasional small nucleolar vacuoles, appearing as clear areas of variable size, were observed during active GC export into the cytoplasm. Treatment with the emulsifier did not alter nucleolar ultrastructure. In contrast, SEO exposure led to evident reorganization: fibrillar centers were frequently surrounded by reduced amounts of DFC, while areas normally occupied by GC contained prominent clear spaces, suggestive of decreased preribosomal subunit content (Figure 14C, D). Moreover, large nucleolar vacuoles appeared within the

676 nucleolar core (Figure 14C), further emphasizing the disruptive impact of SEO on
677 nucleolar architecture.



678
679 *Figure 14. Ultrastructure of the nucleolus in root meristem cells of V. faba (A, B) and B. napus (B, D) after 24 h of seedling*
680 *incubation in water – Control (A, B), or emulsified SEO at the IC₅₀ concentration (C, D); DFC – dense fibrillar component,*
681 *FC – fibrillar center, GC – granular component, V – vacuole. Scale bar: 1 μm.*

682

3. Discussion

Despite continuous progress in research on the effects of essential oils (EOs) on plant cells, their precise mechanisms of action remain insufficiently understood, largely due to the intrinsic complexity of EO mixtures. When EOs are considered stress-inducing agents in plant development, a unifying feature of their action is the generation of reactive oxygen species (Werrie *et al.*, 2020; Wróblewski, Piotrowska-Niczyporuk, *et al.*, 2025). Our previous studies demonstrated that SEO, at an IC_{50} concentration precisely determined for each tested plant species, induces oxidative stress through extensive ROS production. This process does not abolish the antioxidant system composed of enzymatic and non-enzymatic components, but instead stimulates its activity (Wróblewski, Krajewski, *et al.*, 2025; Wróblewski, Piotrowska-Niczyporuk, *et al.*, 2025). While excessive ROS can disrupt cellular homeostasis and damage essential structures, moderate ROS levels act as secondary messengers in stress signaling, activating multiple pathways (Huang *et al.*, 2019; Werrie *et al.*, 2020). Stress sensing and response in plants requires a coordinated interplay within a complex signaling network that integrates perception, transduction, and effector mechanisms. This network enables plants to detect harmful stimuli, initiate repair and defense responses, and synchronize these processes with metabolic regulation, ensuring cellular homeostasis is either preserved or restored (Chiatante *et al.*, 2021; Li and Zhao, 2024; Porter *et al.*, 2023; Saijo and Loo, 2020).

Among the multiple signaling systems orchestrating plant responses to metabolic status and environmental stress, two stand out as particularly relevant. The sugar-sensing pathway mediated by hexokinase (HXK), together with sucrose synthase (SuSy), regulates carbohydrate allocation and ensures a steady supply of hexoses for both metabolism and signaling. In parallel, the mitogen-activated protein kinase (MAPK) cascade, activated by upstream kinases such as MAPK kinases (MKKs), integrates diverse abiotic stress signals. Both systems function as central hubs that connect metabolic status with external stimuli, including extreme temperatures, salinity, drought, heavy metals, and radiation, thereby initiating adaptive responses (Granot *et al.*, 2014; Jagodzick *et al.*, 2018; Jeandet *et al.*, 2022; Moore *et al.*, 2003; Sakr *et al.*, 2018; Sinha *et al.*, 2011; Taj *et al.*, 2010). Once triggered, the phosphorylation cascade transmits signals

to the nucleus, where it regulates histone-modifying enzymes, chromatin-associated proteins, and transcription factors, thereby modulating the accessibility of stress-responsive genes (Latrasse *et al.*, 2017).

As part of these integrated responses, plant cells initiate a wide spectrum of epigenetic modifications, such as DNA methylation and post-translational histone modifications (PTMs; acetylation, methylation, phosphorylation), particularly affecting H3 and H4 (Miryeganeh, 2021). These modifications act in cross-talk networks that fine-tune chromatin architecture, balancing structural stability with dynamic reorganization. By adjusting gene expression without altering DNA sequence, they provide a versatile regulatory layer essential for plant adaptation to fluctuating and adverse conditions (Jiang *et al.*, 2020; Ramos-Cruz *et al.*, 2021; Shi *et al.*, 2024).

3.1. Suppression of HXK activity and transcriptional response of HXK and SuSy under SEO treatment, with ultrastructural alterations in mitochondria and plastids

In the present study, active HXK was localized in the cytoplasm and in foci corresponding to mitochondria and plastids in root meristem cells of *V. faba* and *B. napus*. Such localization of HXKs agrees with earlier findings in various plant species, including *V. faba* (Polit and Ciereszko, 2009)], *Physcomitrella patens* (Olsson *et al.*, 2003), *Oryza sativa* (J.,-I., Cho *et al.*, 2006), or *Spinacia oleracea* (Wiese *et al.*, 1999). The colocalization of HXK activity with organelles reflects its function in coordinating sugar metabolism, with broader cellular signaling networks, including cell cycle regulation (Polit and Ciereszko, 2009). Moreover, the nuclear presence of HXK1, reported for AtHXK1 in *A. thaliana* (Y.,-H., Cho *et al.*, 2006) supports the idea that HXK may act directly in transcriptional control by forming part of signaling complexes that couple metabolic status with gene expression (Y.,-H., Cho *et al.*, 2006).

Following exposure to SEO at IC₅₀ concentrations, a marked reduction in HXK activity was observed both in organelle-associated regions and the cytoplasm, suggesting that either the excessive ROS generated under SEO stress (Wróblewski, Krajewski, *et al.*, 2025) or specific phytochemicals in the oil (Szczęblewski *et al.*, 2022) impair enzyme function. Comparable stress-induced inhibition of HXK has been described under drought,

underscoring the sensitivity of glycolytic regulation to redox imbalance (Wu *et al.*, 2023). ROS-mediated HXK inactivation has also been reported in animals via oxidative modification of thiol groups (Barrett *et al.*, 2012). In addition, phenolic constituents present in essential oils can interfere with glycolytic enzymes, which has been widely proven in cancers (Bakrim *et al.*, 2022; Zhao *et al.*, 2022). On the other hand, the reduced HXK activity may result from damage to the outer membranes of mitochondria or plastids, as observed in our ultrastructural analyses. For its full activity, HXK requires proper integration with the channel protein VDAC in the intact mitochondrial membrane, which provides privileged access to ATP; disruption of this interaction leads to a decline in enzyme activity (Camara *et al.*, 2017; Rostovtseva *et al.*, 2021). In line with this, the reduced starch content observed in plastids following SEO treatment may result from impaired HXK activity, limiting the availability of phosphorylated sugars for starch synthesis but also from stress-induced reallocation of carbon resources away from storage compounds toward energy production and defense metabolism (Mmbando, 2024). Moreover, ultrastructural alterations of plastids, including elongation and the presence of prolamellar bodies, are consistent with a reprogrammed metabolic state less favorable for starch deposition. Regardless of the underlying mechanism of HXK inhibition, such inhibition likely generates a metabolic signal to the nucleus, indicating a shortage of readily utilizable sugars for energy production. In meristematic cells, where proliferation requires high metabolic flux, this signal could redirect transcriptional programs away from cell cycle progression toward energy conservation and stress survival strategies (Rawat and Laxmi, 2024). Indeed, transcriptional responses indicate that, in the face of suppressed HXK activity, cells activate compensatory pathways to maintain metabolic flux. While HXK transcript levels showed only a slight increase under SEO treatment compared with control seedlings, SuSy expression was strongly upregulated, exceeding a threefold increase relative to the control. In light of mitochondrial damage and reduced HXK activity, the upregulation of SuSy may support mitochondrial respiration, supplying energy for coping with adverse conditions. Alternatively, enhanced SuSy expression could promote UDP-glucose production, potentially utilized for callose deposition in cell walls to mitigate increased permeability resulting from membrane damage (Wang *et al.*, 2021). These findings are consistent with RNA-seq data from eugenol-treated Chinese cabbage, where genes associated with

callose synthesis were upregulated (Hsieh *et al.*, 2024). Overall, this transcriptional profile indicates that, despite the suppression of HXK activity, metabolic flux through sucrose cleavage pathways may be enhanced, ensuring a continued supply of hexoses for signaling and stress-related metabolism, and representing an adaptive response that buffers the metabolic and structural perturbations caused by SEO. Hence, our results suggest that SEO not only induces oxidative stress but also directly targets metabolic regulators such as HXK, linking metabolic perturbations to stress signaling and adaptive responses.

3.2. SEO-induced oxidative stress and activation of MAPK signaling, including MKK transcriptional response

In our experiments, 24 h of seedling incubation with SEO at IC₅₀ concentration resulted in pronounced activation of nuclear MAPKs, detected in *V. faba* by immunocytochemical staining and in *B. napus* by Western blotting. The signal corresponded to MPK3 and MPK6 (plant homologs of mammalian p44/42 MAPKs, also known as Erk1/2), recognized by anti-phospho-p44/42 (Thr202/Tyr204) antibodies. In *V. faba*, MAPK activation appeared as distinct nuclear foci, whereas in *B. napus*, no individual foci were distinguishable, likely due to interspecific differences such as genome size and nuclear architecture (*V. faba* exhibits a reticulate nucleus with chromocenters, whereas *B. napus* has an euchromocentric type). This nuclear activation was accompanied by an increase in MKK transcript levels, indicating that SEO not only promotes post-translational MAPK phosphorylation but also enhances upstream transcriptional inputs into the cascade.

Although studies directly examining SEO-mediated MAPK activation in plants are lacking, similar effects have been documented for phenylpropanoids, the compound class including asarones present in SEO, which can activate MAPK pathways in animals such as methyl eugenol (Kuang *et al.*, 2023) and safrole (Ni *et al.*, 2020) or even inhibit this pathway, as was proven for β -asarone (Huang *et al.*, 2016).

Abundant evidence also shows that ROS generated under abiotic and biotic stresses activate MPK3 and MPK6. For instance, H₂O₂ treatment rapidly induces MPK3/MPK6 phosphorylation in *A. thaliana* (Kovtun *et al.*, 2000), ozone and H₂O₂ activate SIPK/WIPK

(homologs of MPK6/MPK3) in *Nicotiana tabacum* (Samuel *et al.*, 2005), and pathogen-induced ROS trigger MAPK cascades in *A. thaliana* (He *et al.*, 2020; Nakagami *et al.*, 2006).

Activated MAPKs influence epigenetic regulation by modulating the activity of chromatin-modifying enzymes, histone writers and erasers, and remodelers, thereby shaping transcriptional responses (Bigeard and Hirt, 2018). In humans, MAPK-mediated phosphorylation of histone acetyltransferase p300 has been linked to chromatin relaxation, facilitating transcription activation (Chang *et al.*, 2021). P300 homologs have been identified in *A. thaliana* (Fina *et al.*, 2017), although the role of MAPK in p300 phosphorylation has not been studied.

Based on our findings, combined with the strong ROS accumulation reported previously under identical SEO treatment conditions (Wróblewski, Krajewski, *et al.*, 2025; Wróblewski, Piotrowska-Niczyporuk, *et al.*, 2025), we propose that SEO activates MAPK signaling through ROS. The concomitant increase in MKK transcript levels further indicates that the entire MAPK cascade was engaged, reinforcing the phosphorylation-based activation of nuclear MAPKs. This activation subsequently initiates downstream epigenetic reprogramming. Such chromatin-level regulation may not only protect DNA from endonuclease-mediated degradation (Genchi *et al.*, 2020) but also enable adaptive transcriptional changes that enhance stress tolerance (Bigeard and Hirt, 2018; Latrasse *et al.*, 2017).

3.3. Increase in DNA methylation as a component of the epigenetic response to SEO-induced stress

Building upon the MAPK-mediated signaling events described above, another essential layer of the SEO-induced stress response is the regulation of DNA methylation. In general, methylation of gene promoter regions leads to chromatin condensation, thereby restricting the access of transcription factors and ultimately repressing gene expression (Kim *et al.*, 2021; Luan *et al.*, 2020).

Our analyses, based on immunofluorescent detection of 5-methylcytosine (5-MeC), revealed pronounced DNA hypermethylation within the nuclear chromatin of root meristematic cells in both *V. faba* and *B. napus* following exposure to SEO at IC₅₀ concentrations. This was accompanied by an increased proportion of condensed

chromatin domains within nuclei of all four examined species, as demonstrated by ultrastructural observations. These phenomena coincided with elevated activity of nuclear MAPKs, suggesting a possible functional link between stress-induced signaling cascades and epigenetic modulation of the transcriptional response. Comparable hypermethylation triggered by ROS or heavy metals has been reported in *Glycine max* L. and *Raphanus sativus* L. (Sun *et al.*, 2021; Tang *et al.*, 2021), supporting the notion that such epigenetic adjustments represent a widespread mechanism in plants. These modifications may reflect a dual adaptive strategy: first, to safeguard genome integrity threatened by SEO-induced reactive oxygen species (ROS), known to generate DNA strand breaks, as demonstrated in our earlier studies (Wróblewski, Krajewski, *et al.*, 2025), and second, to fine-tune gene expression patterns in ways that promote cellular survival under stress.

3.4. Epigenetic landscape shaped by histone modifications under SEO-induced stress

Like DNA methylation, post-translational modifications (PTMs) of histones also regulate chromatin accessibility, thereby controlling essential nuclear processes such as DNA replication, repair, and transcription, both during normal physiological development and in response to stress. Under abiotic stresses such as drought, salinity, temperature extremes, or exposure to heavy metals, histone marks undergo dynamic reconfiguration that enables chromatin remodeling and transcriptional reprogramming (Gallusci *et al.*, 2023). However, there is no universal pattern across plant species in either the type or the extent of histone modifications; their dynamics depend on the specific stressor, the plant species, and even individual variability (Gupta and Salgotra, 2022; Susek *et al.*, 2017). Our findings demonstrate that SEO, at an IC₅₀ concentration, significantly alters histone methylation, acetylation, and phosphorylation patterns in the root meristematic cells of *V. faba* (Fabaceae) and *B. napus* (Brassicaceae), while also revealing some species-specific differences. This underscores the pivotal role of epigenetic regulation in orchestrating plant responses to SEO.

3.4.1. H3K4Me2

Methylation of histone H3 at lysine 4 (H3K4) is one of the most conserved histone modifications, occurring in mono-, di-, and trimethylated states. In *A. thaliana*, H3K4Me1

is present in approximately 80% of genes, H3K4Me2 in about 84%, and H3K4Me3 in roughly 62% of the genome (Xiao *et al.*, 2022). The distribution pattern of these modifications is characteristic: H3K4Me1 is predominantly localized within coding regions, whereas H3K4Me2 and H3K4Me3 are enriched in promoter regions, close to transcription start sites. A similar organization has been reported not only in plants (Oya *et al.*, 2022; Zhang *et al.*, 2009), but also in yeast (Pokholok *et al.*, 2005) and animals (Barski *et al.*, 2007), underscoring the strong evolutionary conservation of this histone mark.

Although H3K4 methylation is generally regarded as a marker of active chromatin, the role of H3K4Me2 remains controversial (Barski *et al.*, 2007; Oya *et al.*, 2022; Zhang *et al.*, 2009). In plants, increasing evidence suggests that H3K4Me2 is more often associated with transcriptional repression (Ishihara *et al.*, 2019; Kim *et al.*, 2012; Liu *et al.*, 2019; Mori *et al.*, 2023; Oya *et al.*, 2022; Pereira *et al.*, 2025). By contrast, in mammals, H3K4Me2 plays a protective role by preventing *de novo* DNA methylation at active promoters. This is achieved by blocking the binding of DNMT3L, a cofactor of the DNMT3A/B methyltransferases, which preferentially interact with unmodified H3K4 residues (Meissner *et al.*, 2008; Ooi *et al.*, 2007). Consequently, H3K4Me2 acts as an epigenetic barrier that safeguards genes against unwanted transcriptional silencing (Fu *et al.*, 2020; Li *et al.*, 2021).

Our results clearly show that SEO-induced stress markedly increases H3K4Me2 levels in root meristem cells of *V. faba* and *B. napus*. This effect coincided with a global rise in DNA methylation and a decline in transcriptional activity, suggesting that SEO interferes with H3K4-dependent epigenetic regulation in a manner that promotes transcriptional repression. Similar patterns, i.e., an accumulation of H3K4Me2, have been described in *Zea mays* under drought and salt stress (Kamal *et al.*, 2021). Conversely, a decrease in H3K4Me2/3 was reported in *Camellia sinensis* subjected to drought stress (Chen *et al.*, 2021), as well as in meristematic cells of *V. faba* exposed to cadmium stress (Gocek-Szczurtek *et al.* our unpublished data). Collectively, these findings indicate that the role of H3K4Me2 in stress responses is both species- and stressor-dependent. Under SEO exposure, this modification is strongly enhanced and coincides with increased DNA methylation and a global reduction of transcriptional activity.

3.4.2. H3T45Ph

Phosphorylation of histone H3 at threonine 45 (H3T45Ph) represents an important structural modification that influences chromatin architecture by regulating nucleosome dynamics. The T45 residue, positioned in close proximity to Y41 and R42 at the DNA entry/exit site of the nucleosome, is part of the so-called ‘H3 latch’, which ensures the stability of DNA–histone interactions (Armeev *et al.*, 2021). Addition of a phosphate group at this position alters the local electrostatic environment, reduces the affinity of DNA for the histone octamer, and facilitates partial DNA unwrapping, thereby enhancing chromatin flexibility (Brehove *et al.*, 2015). Although plant-specific data on this modification are limited, experimental evidence from *Saccharomyces cerevisiae* (Baker *et al.*, 2010; Hurd and Nelson, 2009) and from mammalian cells (Lee *et al.*, 2015) highlights its involvement in replication, DNA repair, and the DNA damage response and it cannot be excluded that, by promoting chromatin relaxation, it may also facilitate transcription.

In our experiments, exposure to SEO caused a pronounced decrease in H3T45Ph levels in root meristem cells, with the reduction being particularly evident in *B. napus*. Given the established role of this modification in maintaining accessible chromatin, its downregulation aligns with the observed increase in DNA methylation and the global suppression of transcriptional activity. Furthermore, our previous studies demonstrated that SEO-induced stress was associated with DNA damage, prolonged replication, and an accumulation of cells in late S phase, corresponding to the replication of condensed heterochromatin (Wróblewski, Krajewski, *et al.*, 2025). These effects were most likely linked to impaired completion of DNA repair in these regions. The reduced abundance of H3T45Ph under SEO treatment likely exacerbates these problems by restricting chromatin accessibility for repair and replication machineries, thereby reinforcing the observed replication stress phenotype. Interestingly, the less pronounced decrease in H3T45 phosphorylation observed in *V. faba* compared to control, relative to the stronger reduction detected in *B. napus*, suggests that the representative of Fabaceae (considered a protected crop species) may retain a higher capacity for DNA repair under SEO-induced stress. In contrast, *B. napus*, a species deliberately targeted for suppression in agricultural systems, appears to be more vulnerable due to its limited

ability to maintain this chromatin modification. Consistent with this interpretation, we observed a higher proportion of meristematic *B. napus* cells residing in S phase compared to control, yet exhibiting reduced replication dynamics and stalling in the late stages of DNA synthesis (Wróblewski, Krajewski, *et al.*, 2025).

3.4.3. H4K5Ac

A comparable conclusion emerges from the analysis of histone H4 acetylation at lysine 5 (H4K5Ac), a modification that under physiological conditions occurs in both euchromatic and heterochromatic domains of interphase nuclei, where it contributes to chromatin relaxation required for transcription and replication (Žabka *et al.*, 2021). Immunocytochemical assays demonstrated that SEO exposure led to a pronounced reduction in H4K5Ac signal intensity. The loss of acetylation coincided with decreased replication activity and the accumulation of DNA damage, as evidenced by the presence of γ -H2AX (Wróblewski, Krajewski, *et al.*, 2025), as well as with the overall decline in transcriptional activity documented in this study. Similar effects were also recorded in our previous analyses of *V. faba* meristematic cells exposed to cadmium (Gocek-Szczurtek *et al.*, our unpublished data). Collectively, these findings indicate the induction of a broad chromatin-silencing response under stress conditions, including SEO treatment. A reduction in H4K5Ac levels has also been reported in *Hibiscus cannabinus* subjected to salt (NaCl) and polyethylene glycol (PEG) stress (Wei *et al.*, 2019).

It is also worth noting that H4K5 acetylation has been linked to transcriptional ‘priming’, i.e., the poising of genes for rapid activation upon environmental challenge (Park *et al.*, 2013). This concept helps reconcile our observation of strong H4K5Ac signals in a subset of nuclei with prominent nucleolar labeling, despite an overall decline of this modification in SEO-treated cells. The nucleolus, responsible for rDNA transcription and ribosome biogenesis, is highly stress-sensitive and undergoes chromatin reorganization and transcriptional modulation in response to stress (Lewinska *et al.*, 2010; Sapio *et al.*, 2021). In this context, focal H4K5Ac enrichment within or adjacent to nucleolar organizer regions likely reflects an adaptive attempt to sustain rDNA transcription and ribosome production under SEO-induced oxidative stress and perturbed cellular homeostasis. The resulting nucleolar pattern may also indicate selective preservation of accessibility at genomic loci that are critical for stress mitigation.

Against a background of increased global DNA methylation and elevated H3K4Me2, a mark frequently associated with repression in plants, the persistence of localized H4K5Ac is plausibly interpreted as an exception to genome-wide silencing, maintaining the expression of genes essential for survival under stress. Consistent with this interpretation, heat stress in *Zea mays* was shown to induce nucleolar reorganization accompanied by histone acetylation within the 45S rDNA region, correlating with enhanced pre-rRNA synthesis (Yue *et al.*, 2021). Similarly, in *Brassica rapa* subsp. *chinensis* exposed to cadmium, increased H4K5Ac at the 45S rDNA promoter was associated with activation of rRNA transcription (Xiang *et al.*, 2024).

3.4.4. H3K9Ac

Despite the overall shift in the balance toward epigenetic modifications that promote chromatin condensation - indicating that, despite interspecific differences, cells nonetheless activate a common program of genome protection (e.g., increased levels of H3K4Me2 and decreased levels of H3T45Ph and global H4K5Ac) in response to SEO-induced stress - an increase in specific histone modifications regarded as markers of transcriptional activity under environmental stress conditions was simultaneously observed. One such modification is acetylation of histone H3 at lysine 9 (H3K9Ac), whose levels rise in the promoter regions of genes activated in response to abiotic stress (Song *et al.*, 2016). In the context of oxidative stress induced by SEO, H3K9Ac may participate in ‘transcriptional reprogramming’, enabling the rapid and selective activation of defense-related genes, such as those encoding antioxidant proteins as well as genes involved in carbohydrate metabolism and energy supply, despite a background of global transcriptional repression under fluctuating environmental conditions (Ageeva-Kieferle *et al.*, 2021; Gallusci *et al.*, 2023; Hu *et al.*, 2019). Consistent with this role, stress-induced increases in H3K9Ac have been reported across different plant systems. For instance, (Wang *et al.*, 2015) showed that heat stress triggered elevated H3K9Ac levels along with reduced histone methylation, while in *A. thaliana* this modification was enriched in the promoters of genes such as *AtMYB29* and *AtGST1*, correlating with their transcriptional induction (Zheng *et al.*, 2016). Similarly, in *O. sativa*, severe salt stress led to a pronounced rise in H3K9Ac in seedling leaves, particularly at the promoter regions of salt-responsive genes (Chang *et al.*, 2024). Taken together, these findings indicate that

H3K9Ac can function as a local mark of active genetic loci, even against the background of global chromatin condensation observed in nuclei following exposure to SEO.

3.4.5. H3K56Ac

SEO-induced stress was also associated with changes in the levels of histone H3 lysine 56 acetylation (H3K56Ac), a modification widely recognized as a marker of cellular responses to DNA damage. Although this mechanism has been thoroughly characterized in yeast, its role in plant cells remains relatively poorly understood (Aricthota *et al.*, 2022). H3K56Ac facilitates local chromatin relaxation, promotes DNA strand separation, and enables the recruitment of DNA repair complexes (Masumoto *et al.*, 2005). Moreover, this modification is incorporated into newly synthesized histone H3 molecules during DNA replication (Duan *et al.*, 2025).

In the present study, exposure of meristematic cells to SEO altered the acetylation profile of H3K56. Instead of uniform labeling across the chromatin, strongly fluorescent foci became more prominent, most likely corresponding to sites of DNA damage. Such a pattern should be interpreted as part of the cellular response to compromised genome integrity, consistent with earlier reports highlighting the genotoxic effects of SEO (Wróblewski, Krajewski, *et al.*, 2025). The importance of H3K56Ac for cell survival is further underscored by studies in *S. cerevisiae*, where mutation of lysine 56, preventing its acetylation, resulted in a dramatic decrease in viability. These mutant cells displayed extreme sensitivity to DNA damage and replication stress (Ge *et al.*, 2013). Collectively, these findings indicate that H3K56Ac plays a crucial role in maintaining genome stability and mediating adaptive responses to environmental stress, including in plant cells.

Taken together, the stress-induced increases in H3K9Ac and focal enrichment of H3K56Ac suggest that, despite genome-wide transcriptional repression and chromatin condensation, plant cells maintain local chromatin flexibility at specific loci to enable both activation of defense-related genes and efficient DNA repair under SEO-induced stress.

3.4.6. Histone-modifying enzyme

Although the epigenetic alterations observed in our study, such as changes in DNA methylation and histone phosphorylation, acetylation, and methylation, can be

convincingly explained as the downstream effect of stress-related signaling cascades reaching the nucleus, an alternative explanation should also be considered. It cannot be excluded that part of these shifts may result not solely from signaling-driven regulation but also from direct interference of SEO constituents with the enzymes responsible for establishing or removing epigenetic marks. Many of these enzymes contain reactive cysteine residues in their catalytic centers or rely on thiol-based redox regulation to maintain activity and structural integrity (Genchi *et al.*, 2020). Phenylpropanoids such as α - and β -asarone, the dominant components of SEO, possess electrophilic properties and can undergo bioactivation into reactive intermediates capable of covalently modifying protein thiols (Chen *et al.*, 2023). Indeed, many essential oils rich in phenylpropanoids (e.g., eugenol, isoeugenol) have a documented ability to inactivate cysteine-dependent enzymes through –SH group modification (Ahn *et al.*, 2020; Chen *et al.*, 2023). Such interactions could, in principle, compromise the activity of DNA methyltransferases, histone acetyltransferases, deacetylases, or kinases and phosphatases that rely on cysteine residues. Moreover, electrophilic compounds may promote oxidation of thiol groups to disulfides, leading to conformational changes or functional inactivation of critical enzymes involved in chromatin remodeling (Li *et al.*, 2025). This opens the possibility that at least part of the epigenetic reprogramming detected in root meristematic cells could stem from direct chemical inactivation of chromatin-modifying enzymes rather than being entirely orchestrated through upstream signaling pathways. Nevertheless, the highly coordinated nature of the changes observed (manifested by repression of global transcription, selective activation of stress-responsive programs, and adjustment to DNA damage) strongly suggests a controlled and adaptive nuclear response. While the contribution of direct enzyme damage cannot be fully ruled out, the logic and consistency of the epigenetic patterning point more convincingly toward regulation rather than stochastic loss of enzymatic function.

Taken together, the observed shifts in DNA methylation and histone modification patterns, accompanied by MAPK activation and HXK-related signaling, indicate that exposure to SEO at IC_{50} concentrations does not collapse the cellular machinery but instead elicits a coordinated protective program in meristematic cells. Although the magnitude of individual responses varied slightly among the studied species, the

underlying regulatory framework remained largely conserved. This suggests that SEO acts as a potent trigger of stress-adaptive signaling and epigenetic reprogramming, reinforcing the plant's intrinsic defense capacity rather than irreversibly impairing it.

An intriguing next step will be to determine how this protective program performs when SEO is applied at herbicidal concentrations - specifically, at doses safe for Fabaceae but exceeding the IC_{50} threshold for Brassicaceae. Such experiments will be essential to evaluate whether the stress-adaptive framework observed here remains effective under agronomically relevant conditions.

Overall, these findings support the view that SEO functions not as a nonspecific cytotoxin but rather as a complex stressor that reshapes nuclear responses in a regulated manner. The possibility that epigenetic flexibility contributes to interspecific differences in tolerance - such as the relative resilience of Fabaceae versus Brassicaceae - deserves further investigation, particularly in the context of developing SEO-based bioherbicide.

3.5. Inhibition of transcriptional activity as an effect of SEO stress

Transcription is one of the key processes regulating cellular function, determining both proliferation and development under physiologically favorable as well as stressful and changing environmental conditions. Epigenetic modifications, which serve to protect the genetic material from damage and regulate DNA accessibility to the transcriptional machinery, play a central role in chromatin reorganization and its adjustment to prevailing conditions (Pucholt *et al.*, 2015; Song *et al.*, 2016; Wang *et al.*, 2023). Importantly, transcriptional regulation is not a uniform process. Within a single cell, activating and repressive modifications may coexist, reflecting the complex and dynamic nature of chromatin-mediated control of gene expression.

In the present study, using the transcriptional activity marker EU in combination with fluorescence microscopy, a pronounced decrease in RNA synthesis was observed in SEO-treated cells, both in the nucleus and in the nucleolus. This interpretation is further supported by ultrastructural observations. Electron micrographs revealed that SEO exposure caused striking alterations in nucleolar organization. The nucleolus, a dynamic nuclear structure responsible for ribosome biogenesis, serves as a sensitive indicator of cellular activity and stress responses (Lewinska *et al.*, 2010). In treated cells, nucleoli

exhibited impoverishment of both the fibrillar and granular components, as evidenced by the presence of large nucleolar vacuoles, loosely dispersed granules, and a segregated appearance of fibrillar and granular domains. Such features are characteristic of reduced rRNA transcription and impaired nucleolar function. Moreover, global repression of transcription, driven by enhanced DNA methylation and the resulting chromatin condensation, was accompanied by a decrease in the number of nuclear pores visible in the nuclear envelope. This reduction may reflect the lower demand for nucleocytoplasmic transport under conditions of limited transcript production and suggests an adaptive adjustment of nuclear architecture to reduced transcriptional output (Wagner *et al.*, 1990). It should be emphasized, however, that a decrease in overall transcription does not imply uniform downregulation of all genes. Stress conditions frequently cause a general decline in transcriptional and translational activity while simultaneously enhancing the expression of stress-responsive genes (Duncan and Hershey, 1987). For example, histone acetylation at residues such as H3K9 is associated with activation of defense-related genes, whereas DNA methylation may transiently repress genes involved in growth and metabolism, thereby reallocating energy toward protective mechanisms (Hinnebusch, 1994). This dual regulatory strategy is well illustrated by an RNA-Seq meta-analysis in *O. sativa*, which demonstrated repression of photosynthetic pathways alongside induction of phytohormone-related genes under biotic and abiotic stresses (Cohen and Leach, 2019). Similarly, Kovalchuk *et al.*, (2005) reported that chronic exposure of plants to Cd (50 μ M) induced extensive transcriptional reprogramming, with 65 genes upregulated and 338 downregulated. In *Hordeum vulgare* L., Cd exposure at 80 μ M resulted in a general decline in transcript abundance in both root and shoot tissues (Kintlová *et al.*, 2021). Comparable effects have also been observed in animal systems exposed to essential oil constituents (Firmino *et al.*, 2021).

Taken together, these observations indicate that SEO does not merely suppress transcription globally but rather orchestrates a nuanced reprogramming of gene expression, balancing genome-wide repression with selective activation of stress-responsive genes to optimize cellular adaptation under adverse conditions.

4. Conclusions

SEO either directly or indirectly, through enhanced ROS generation, disrupts hexokinase (HXK)-dependent metabolic signaling by reducing its enzymatic activity, consequently decreasing the availability of sugar metabolites for downstream metabolic processes. This effect may be associated with structural damage to membranes, as revealed by electron microscopy. The resulting signal reflects an energy deficit that could constrain the proliferative capacity of meristematic cells.

In parallel, SEO also stimulates signal transduction to the nucleus via the MAPK cascade, as evidenced by the increased presence of phosphorylated MPK3 and MPK6 (plant homologs of mammalian p44/42 MAPKs, also known as Erk1/2) within the chromatin compartment, suggesting their potential role in modulating chromatin function and transcriptional regulation.

Consequently, signals transmitted to the nucleus in response to SEO exposure induce chromatin remodeling aimed at maintaining homeostasis and ensuring cell survival. This process is manifested by a global repression of transcription due to energy limitation and involves epigenetic modifications that favor chromatin condensation, including increased DNA methylation, elevated H3K4Me2, decreased H3T45Ph, and a global reduction in H4K5Ac.

Simultaneously, SEO-induced stress and the associated DNA damage trigger adaptive histone modifications. These include local (nucleolar) H4K5 and H3K9 acetylation, enabling the selective activation of stress-responsive genes, as well as H3K56 acetylation, which facilitates localized chromatin relaxation in regions necessary for efficient DNA repair.

In response to HXK inhibition under SEO-induced stress, meristematic cells appear to reprogram transcriptional activity by upregulating alternative metabolic routes. This includes enhanced transcription of sucrose synthase (SuSy) to supply UDP-glucose in response to reduced hexose availability from HXK-dependent pathways, while HXK transcript levels themselves also increase, suggesting an attempt to restore the primary sugar-sensing pathway and maintain carbohydrate flux under stress conditions.

The observed enhancement of MAPK signaling under SEO stress is likely supported not only by increased phosphorylation of MPK3/MPK6 but also by elevated transcription of upstream MAPK kinases (MKKs). This coordinated upregulation suggests that meristematic cells reinforce the entire MAPK cascade, ensuring a robust stress-responsive signaling network that transmits environmental and metabolic cues to the nucleus.

SEO does not disrupt the function of the interphase chromatin-remodeling machinery; rather, it activates it in a coordinated manner. The observed epigenetic changes indicate a controlled cellular response, rather than a random dysregulation of remodeling processes.

Despite minor interspecific differences in the levels and distribution patterns of specific epigenetic marks, the mechanism by which SEO modulates the structural and functional properties of chromatin appears to be conserved among the examined species from the Fabaceae and Brassicaceae families, with Fabaceae exhibiting greater adaptive plasticity in response to stress while the overall regulatory framework remains largely similar.

Taken together, these findings provide new mechanistic insights into how essential oils interfere with nuclear signaling and epigenetic regulation. They also highlight the potential of SEO as a bioherbicidal agent capable of impairing growth and proliferation, offering a promising tool for sustainable weed management strategies.

5. Materials and methods

5.1. Plant material

Seeds of 4 plant species: faba bean (*Vicia faba* L. subsp. *minor*, cv. Bobas; Danko, HR Sp. z o. o. Choryń, Poland), yellow lupine (*Lupinus luteus* L., cv. Baryt; PHR Sp. z o. o. Tulce, Poland), rapeseed (*Brassica napus* L. cv. Markus; HR Strzelce Sp. z o. o. Strzelce, Poland), and thale cress (*Arabidopsis thaliana* Col-0 (L.) Heynh.; NASC, Nottingham, UK) were germinated on filter paper moistened with deionized water in Petri dishes for 2–3 days at 23 °C in darkness, except *A. thaliana* germinating in long-day conditions (16/8 h) due to etiolation sensitivity. Seedlings with equal-sized primary roots (about 2 cm *V. faba*, *L. luteus*, or 1 cm *B. napus* and 0.5 cm *A. thaliana*) were selected for further experiments as previously described (Wróblewski, Krajewski, *et al.*, 2025; Wróblewski, Piotrowska-Niczyporuk, *et al.*, 2025).

5.2. Essential oil treatment

Sweet flag essential oil (SEO) (Etja, Elbląg, Poland) was emulsified in a 1:4 (v/v) ratio with a mixture of C14-18 and unsaturated C16-18—mono- and di-ethoxylated glycerides and ethoxylated *B. napus* oil as described previously (Wróblewski, Krajewski, *et al.*, 2025; Wróblewski, Piotrowska-Niczyporuk, *et al.*, 2025). Seedlings were incubated in emulsified SEO diluted in water to obtain corresponding for each species IC₅₀ values: 0.03 % for *V. faba*, 0.025 % for *L. luteus*, 0.01 % for *B. napus*, and 0.005 % for *A. thaliana* (Wróblewski, Piotrowska-Niczyporuk, *et al.*, 2025). The emulsifier and SEO solutions were poured on a filter paper, and the incubation was carried out in tightly sealed with Parafilm Petri dishes, in darkness, at 23 °C for 24 h (Wróblewski, Krajewski, *et al.*, 2025; Wróblewski, Piotrowska-Niczyporuk, *et al.*, 2025). Ten root meristems were examined per species (Control series, emulsifier series - E, and SEO series - O), and four experimental repeats were performed, resulting in a total of n = 40 root meristems per species per treatment.

5.3. Visualization of hexokinase activity

Sectioned roots were fixed in the fixative consisting of 2% paraformaldehyde (Sigma Aldrich), 2 % PVP 40 (Sigma Aldrich), 0.001 M dithiothreitol (Sigma Aldrich) for 1 h at 4°C, rinsed overnight and 5 times before staining procedures to remove soluble

carbohydrates. The reaction medium contained 50 mM 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)-1,3-propanediol buffer (pH 8.0), 6.25 mM MgCl₂, 2.5 mM ATP, 1 mM NAD, 1 U glucose-6-phosphate dehydrogenase (G6PDH), 12.5 mM HEPES-KOH (pH 7.4), 0.25 mM EGTA, 0.25 mM EDTA, 0.025 % BSA, 0.03 % NBT and 0.5 mM glucose to start the assay. In the negative control, glucose or NAD were omitted. The root sections were stained for 1 h at 30°C and rinsed in distilled water to terminate the reaction (Polit and Ciereszko, 2009; Sergeeva and Vreugdenhil, 2002). The principle of the visualization of HXK activity is the coupling of the reduction of NAD to the reduction of nitroblue tetrazolium salt (NBT), resulting in precipitation of the blue formazan salt (Sergeeva and Vreugdenhil, 2002)

5.4. Immunocytochemical detection of phosphorylated MAP kinases and modified histones

Meristematic zones of *V. faba* and *B. napus* primary roots were fixed in 0.01 M PBS-buffered 4% paraformaldehyde (4 °C, 40 min) and macerated with a 0.1 M citrate-buffered enzyme mixture of 2.5 % pectinase, 2.5 % pectolyase, and 2.5 % cellulase (Sigma Aldrich) at 37 °C for 30 min (*V. faba*) or 20 min (*B. napus*), were squashed onto Super Frost Plus microscope slides (Menzel-Gläser) following the procedures described in (Wróblewski, Krajewski, *et al.*, 2025). Microscopic preparations were incubated overnight at 4 °C in darkness and a humidified atmosphere with primary antibodies: Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody, which cross-reacts with plant homologs MPK3 and MPK6 (Cell Signaling Technology, Danvers, Massachusetts, USA), diluted 1:500 in antibody buffer (1% BSA; 0.3% Triton X-100 in 0.01 M PBS); H3K4Me2 monoclonal antibody (Thermo Fisher Scientific) diluted 1:200; H3K56Ac recombinant rabbit monoclonal antibody (3H2) (Thermo Fisher Scientific) diluted 1:200; Anti-Histone H3 (phospho T45) antibody (Abcam, Cambridge, UK) diluted 1:200; Acetyl-Histone H3 (Lys9) (C5B11) Rabbit mAb (Cell Signaling Technology) diluted 1:200; Anti-Histone H4 (Acetyl-Lys5) antibody produced in rabbit (Sigma-Aldrich, St. Louis, MI, USA) diluted 1:200. In the next day, slides were rinsed with 0.01 M PBS and incubated with the Anti-rabbit IgG (H+L), F(ab')₂ Fragment (Alexa Fluor® 488 Conjugate) secondary antibodies, stained with DAPI and embedded in 0.01 M PBS/glycerol mixture (9:1) with 2.3% DABCO according to the procedures described in (Wróblewski, Krajewski, *et al.*, 2025).

5.5. Protein isolation and Western Blot

Proteins from *B. napus* roots were extracted using Pierce Plant Total Protein Extraction Kit (Thermo Fisher Scientific) supplemented with Protease Inhibitor Cocktail (Sigma Aldrich) according to the manufacturer's instructions. Protein concentrations were evaluated using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). 40 µg of proteins were separated on NuPAGE Novex 4-12% Bis-Tris gel and transferred onto 0.45 µm pore size polyvinylidene fluoride (PVDF) membrane. Membranes were incubated with Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody, which cross-reacts with plant homologs MPK3 and MPK6 (Cell Signaling Technology) in a 1:1000 dilution and actin using WesternBreeze™ Chromogenic Kit, anti-rabbit (Thermo Fisher Scientific) according to the manufacturer's instructions. Band analyses were performed using GelAnalyzer 23.1.1 (available at www.gelalyzer.com) by Istvan Lazar Jr., PhD, and Istvan Lazar Sr., PhD, according to the procedures described previously (Wróblewski, Krajewski, *et al.*, 2025).

5.6. Immunocytochemical detection of 5-methylcytosine

Meristematic zones of *V. faba* and *B. napus* primary roots were fixed in 0.01 M PBS-buffered 4% paraformaldehyde (4 °C, 10 min), washed with a cold Tris buffer (10 mM Tris, 10 mM EDTA- Na_2 , 100 mM NaCl; pH 7.2) and cell nuclei were isolated and dropped onto Super Frost Plus microscope slides (Menzel-Gläser, Germany). After air-drying isolated nuclei were treated with 4M HCl for 90 min to partially denature DNA, washed with 0.5% Triton X-100 (Sigma Aldrich, St. Louis, MI, USA) in Tris buffer and incubated overnight at 4°C with primary anti-5-methylcytosine antibodies (5-MetC, Sigma Aldrich) diluted 1:100 in the antibody buffer (1% BSA; 0.3% Triton X-100 in 0.01 M PBS). In the next day, slides were washed with Tris buffer and incubated for 90 min with the Anti-rabbit IgG (H+L), F(ab')₂ Fragment (Alexa Fluor® 488 Conjugate) secondary antibodies (Cell Signaling Technology (Danvers, Massachusetts, USA) diluted 1:500 in antibody buffer, washed with Tris buffer and embedded in 0.01 M PBS/glycerol mixture (9:1) with 2.3% DABCO.

5.7. EU pulse labelling

Seedlings of *V. faba*, *L. luteus*, *B. napus*, *A. thaliana* were pulse labelled by 60 min incubation in a 1 mM 5-ethynyluridine (Thermo Fisher Scientific) in darkness. After incubation, meristematic parts of primary roots were fixed in .01 M PBS-buffered 4%

paraformaldehyde (4 °C, 45 min), washed with PBS and macerated with a 0.1 M citrate-buffered enzyme mixture of 2.5% pectinase, 2.5% pectolyase, and 2.5% cellulase (Sigma Aldrich) at 37 °C for 30 min,), were squashed onto Super Frost Plus microscope slides (Menzel-Gläser), frozen on a dry ice block, washed with PBS and air-dried. Next, the microscopic preparations were permeabilized for 15 min in 0.5% Triton X-100 in PBS. Nascent RNA was visualized using the Click-iT RNA Alexa Fluor 488 Imaging kit according to the manufacturer's protocol. DNA was stained with DAPI and microscopic preparations were embedded in 0.01 M PBS/glycerol mixture (9:1) with 2.3% DABCO

5.8. Ag-NOR staining

Detection of phosphoproteins was performed according to (Howell and Black, 1980) after root meristematic tissue fixation using 0.0125 M Sörensen's phosphate-buffered (pH 7.2) 2% glutaraldehyde solution (Polit, 2009).

5.9. Electron microscopy

Apical root fragments were fixed in 2% glutaraldehyde in 1% cacodylate buffer (pH 7.3) for 3 h at 4 °C and postfixed in 1% OsO₄ in 1% cacodylate buffer (pH 7.3) for 3 h. Next, the root fragments were dehydrated in an ethanol series and embedded in Epon 812 and Spurr's resin. Root fragments were sectioned, and ultrathin fragments were double-stained with uranyl acetate and lead citrate (Polit, 2009; Reynolds, 1963). The sections were examined and photographed in a JEOL JEM 1010 transmission electron microscope.

5.10. Microscopic observations and analyses

Observations were performed with an AxioImager.A1 fluorescence microscope (Zeiss, Jena, Germany) equipped with cyanine 3 (Cy3), green fluorescent protein (GFP), and DAPI filters. All images were captured at the same exposure time using an AxioCam MRc5 CCD camera (Zeiss, Jena, Germany) and using a Nikon Eclipse E600W fluorescence microscope (Nikon, Tokyo, Japan) described previously (Wróblewski, Krajewski, *et al.*, 2025). Quantitative color or fluorescence intensity analyses were made in ImageJ software ver. 1.54p (Schneider *et al.*, 2012) after converting color images into greyscale and expressed in arbitrary units.

5.11. Gene expression analysis

To determine the gene expression, 5 excised roots were snap-frozen in liquid nitrogen and ground using a mortar and pestle into a fine powder. Next, 200 mg of the powder was used for RNA isolation using NucleoSpin RNA Plant and Fungi, Mini kit for RNA from plants and fungi (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. RNA concentration and purity were measured using BioDrop Duo (BioDrop Ltd, Cambridge, UK). In each sample, the A260/A280 ratio was < 2.0. RNA integrity was confirmed by separating 1 µg RNA in agarose gel electrophoresis (1 %) in a 1X Tris-acetate-EDTA (TAE) buffer containing ethidium bromide (1 mg · mL⁻¹) at 110 V for 1,5 h at RT. Gel was visualized (Supplementary figure 1) using ProXima 2750 imaging platform (Isogen Life Sciences, De Meern, The Netherlands). Prior to cDNA synthesis, genomic DNA was removed using RQ1 RNase-Free DNase (Promega, Madison, WI, USA) using 3 µg RNA for each sample. Next, the cDNA synthesis was performed for 2 µg input using Oligo(dT)₁₈ Primer (Thermo Fisher Scientific) and SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific) following the manufacturer's protocol. The obtained cDNA was diluted 100X in nuclease-free water and stored at -20°C.

Quantitative real-time PCR (qPCR) was performed using PowerTrack™ SYBR Green Master Mix for qPCR (Thermo Fisher Scientific) using 1 ng cDNA per 10 µl reaction in 4 technical replicates according to the manufacturer's protocol with minor changes consisting of 300 nM primers in each reaction and increased anneal/extend temperature to 61°C using RotorGene Q 5-plex HRM (QIAGEN, Hilden, Germany). Melting curves (Supplementary figure 2) were generated to verify product specificity. Relative expression levels were determined using the 2^{-ΔΔCt} method (Livak and Schmittgen, 2001) and normalized to the reference gene – cyclophilin (*CYP2*) (Gutierrez *et al.*, 2011). Forward and reverse primers (Supplementary table 1) were designed in Primer3 (Untergasser *et al.*, 2012) for coding sequences (CDS), and primer specificity was verified *in silico* using BLAST (<https://galaxy-web.ipk-gatersleben.de>) against *V. faba* cv. 'Hedin/2' v1 CDS features.

5.12. Statistical analysis

Statistical analyses and visualization of outcomes were performed in GraphPad Prism 10 software. Differences between groups were evaluated using Student's t-test or the Mann-

1312 Whitney test. A p-value ≤ 0.05 was considered statistically significant and marked with
1313 asterisks (*) or hash (#).

1314 **Data statement:**

1315 The data will be made available on request.

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1322 **CRedit authorship contribution statement:**

1323 **Mateusz Wróblewski:** Writing – original draft, Visualization, Validation, Project
1324 administration, Methodology, Investigation, Funding acquisition, Formal analysis,
1325 Conceptualization. **Konrad Krajewski:** Visualization. **Natalia Gocek-Szczurtek:**
1326 Visualization. **Aneta Żabka:** Resources. **Justyna T. Polit:** Writing review & editing,
1327 Validation, Supervision, Methodology, Conceptualization.

1328 **Conflict of interest disclosure:** The authors declare no competing financial interests or
1329 personal relationships that could have appeared to influence the work reported in this
1330 paper.

1331 **Supporting information**

1332 A supporting table and figures are attached to the submission system

1333

1334 Table S1. Forward and reverse primers used in this study

1335 Figure S1. Total RNA electrophoresis in TAE-buffered 1 % agarose gel.

1336 Figure S2. Melting curve analyses of RT-qPCR products

1337

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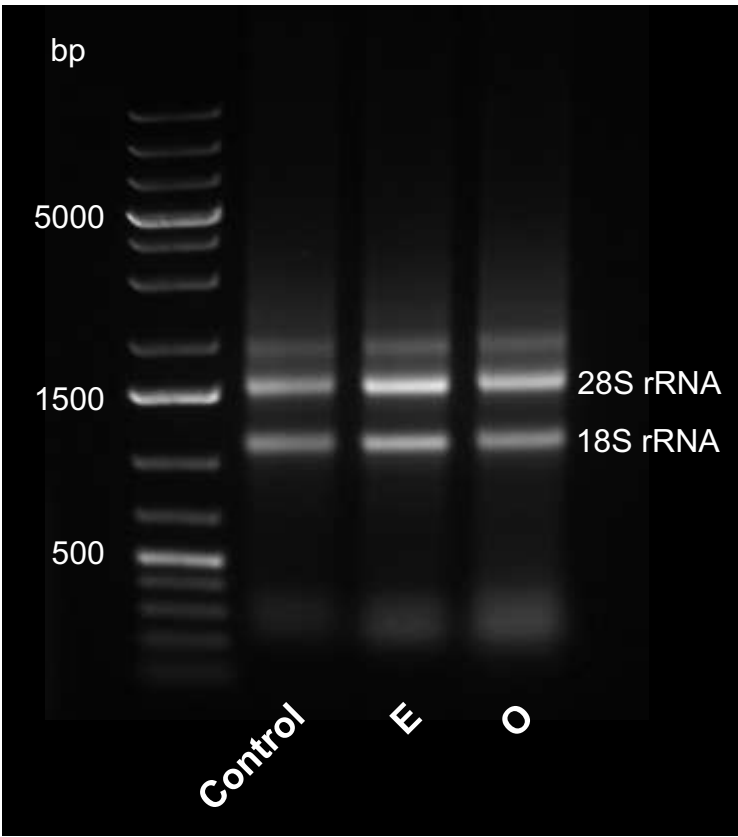
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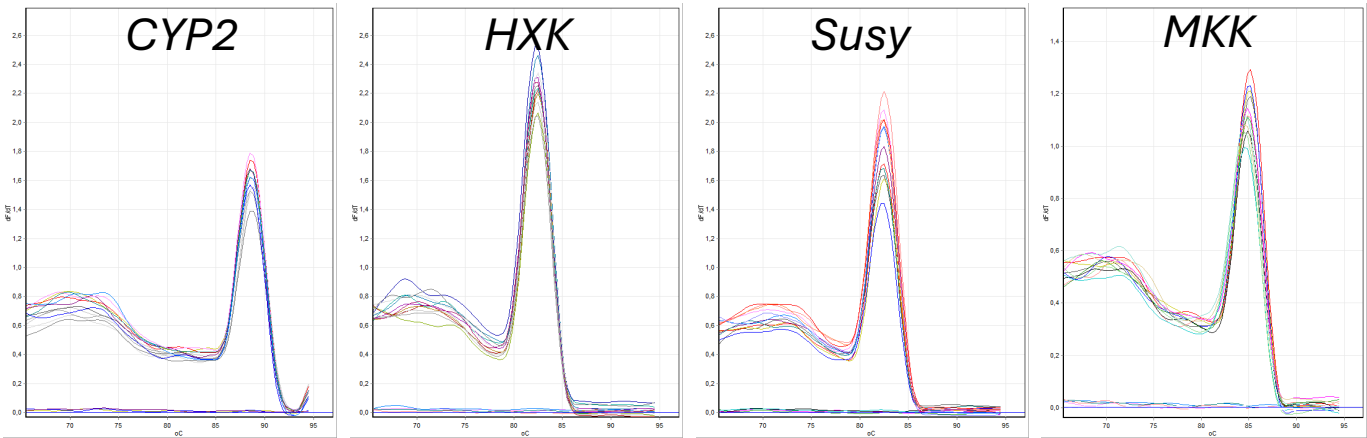
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1 **Supplementary figures**



2 Figure S1. Total RNA electrophoresis in TAE-buffered 1 % agarose gel.



3 Figure S2. Melting curve analyses of RT-qPCR products. The curves represent single
4 peaks for each of the tested gene (*cyclophilin* – *CYP2*; *hexokinase* – *HXK*; *sucrose*
5 *synthase* – *Susy*; *MAP kinase kinase* – *MKK*) and experimental setup. The lines below each
6 curve represent No template control (NTC)

Supplementary table

Table S1. Forward and reverse primers used in this study

Gene	Locus	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon size (bp)	Reference
<i>Cyclophilin (CYP2)</i>	Vfaba.Hedin2.R1.6g063680.1	TGCCGATGTCACTCCCAGAA	CAGCGAACTTGAACCGTAGA		(Gutierrez et al., 2011)
<i>hexokinase protein (HXK)</i>	Vfaba.Hedin2.R1.6g185960.1	GCTAGATCTTGGTGGCACGA	CTTCCGGTTCCGACTCAACA	185	This study
<i>Sucrose synthase isoform (Susy)</i>	Vfaba.Hedin2.R1.4g205320.1	ACCTGCTGAGATCATTGTCCA	ACCGAGAGAGATCTTGTCCCA	139	This study
<i>specificity mitogen activated protein kinase kinase (MKK)</i>	Vfaba.Hedin2.R1.1g172120.1	TTCTCCGATCCACCGAGTCT	TAATTGAGCTGCCGTCCACC	108	This study

Table 1. Key characteristics of plant species used in the study

Latin name	Cultivar/Ecotype	Provider	Key characteristics	References
<i>Vicia faba</i> L. subsp. <i>minor</i>	Bobas	Danko, Choryń Poland	High yields High protein content in seeds Low susceptibility to <i>Bruchus</i> sp. pests High yields	https://danko.pl/odmiany-type/bobas-4/
<i>Lupinus</i> <i>luteus</i> L.	Baryt	PHR, Tulce, Poland	Tolerance to late sowing High protein content High protein content in seeds Low alkaloids level	https://phr.pl/produkty/lubin/lubin-zolty-baryt/
<i>Brassica</i> <i>napus</i> L.	Markus	HR Strzelce, Strzelce, Poland	High yields High resistance to plant diseases Tolerance to periodic droughts High oil content	https://hr-strzelce.pl/markus/
<i>Arabidopsis</i> <i>thaliana</i> (L.) Heynh.)	Col-0	NASC	Natural accession Reference genome	https://arabidopsis.info/InfoPages?template=about_nasc;web_section=arabidopsis



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<https://doi.org/10.1016/j.scienta.2025.114180>.

polegał na:

- współuczestnictwie w: planowaniu pracy eksperymentalnej (obejmującej analizę stresu oksydacyjnego i metabolitów),
- sprawowaniu nadzoru merytorycznego nad tym etapem realizacji badań, w mojej jednostce badawczej, w której odbywał się pobyt doktoranta,
- uczestnictwie w walidacji uzyskanych wyników,
- wprowadzaniu korekt do przygotowywanego manuskryptu.

* - autor korespondujący



.....
Podpis



Raport weryfikacji

Wynik

Wynik przetwarzania

Komunikat błędu

Skrót pliku z podpisem

Nazwa pliku z podpisem

Identyfikator weryfikacji

WAŻNY/PRAWIDŁOWY

Zakończono

Podpis elektroniczny nie został złożony za pomocą kwalifikowanego urządzenia do składania podpisu (QSCD)

4ec7a6e5c4e8c7277c12b105cd8bb79278041617

udział I.Ciereszko podpis elektr.pdf

4DEC2D886F4DACB3A910A937C6071A12ADD6234C

Lista podpisów

Status weryfikacji podpisu

Data i czas wykonania podpisu

Data i czas weryfikacji podpisu

Format podpisu

Poziom podpisu

Certyfikat podpisującego

WAŻNY/PRAWIDŁOWY

2025-09-21 08:30:59 GMT+02:00

2025-09-21 15:57:40 GMT+02:00

PAdES

BES (Podpis w postaci podstawowej)

kwalifikowany

Osoba podpisująca (profil zaufany ePUAP)

Imię i nazwisko

IWONA CIERESZKO

Ścieżka certyfikacji

Certyfikat podpisującego

Walidacja certyfikatu

Status certyfikatu

Wystawiony dla

Wystawiony przez

Ważny od

Ważny do

Data wystawienia CRL/OCSP

Numer seryjny

WAŻNY/PRAWIDŁOWY

WAŻNY

CN=Minister do spraw informatyzacji - pieczęć podpisu zaufanego,
O=Ministerstwo Cyfryzacji, C=PL

CN=Centrum Kwalifikowane EuroCert, O=EuroCert Sp. z o.o., C=PL,

2024-02-13 10:27:09 GMT+01:00

2027-02-12 10:27:09 GMT+01:00

2025-09-21 07:00:01 GMT+02:00

efd46f5b31b845d08071c5eed94d4eb9d37d

Certyfikat wystawcy

Walidacja certyfikatu

Status certyfikatu

Wystawiony dla

Wystawiony przez

Ważny od

Ważny do

Numer seryjny

WAŻNY/PRAWIDŁOWY

WAŻNY

CN=Centrum Kwalifikowane EuroCert, O=EuroCert Sp. z o.o., C=PL,

CN=Narodowe Centrum Certyfikacji, O=Narodowy Bank Polski, C=PL

2017-02-14 13:26:19 GMT+01:00

2028-02-15 00:59:59 GMT+01:00

1a5734b0d472d251e1d37cfe3d796ac117102490

Łódź, 29.09.2025

mgr Natalia Gocek-Szczurtek
Katedra Cytofizjologii
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

Oświadczenie współautora publikacji

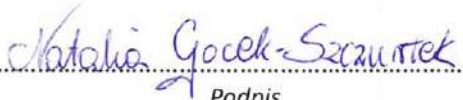
Oświadczam, że mój udział w poniżej wymienionych publikacjach:

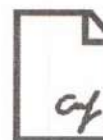
1. Wróblewski, M*.; Piotrowska-Niczyporuk, A.; Ciereszko, I.; **Gocek, N.**; Żabka, A.; Szczęblewski, P.; Sobiech, Ł.; Saja-Garbarz, D.; Polit, J. T. Phytotoxicity and Bioherbicidal Potential of Sweet Flag (*Acorus calamus* L.) Essential Oil on Fabaceae and Brassicaceae Species. *Scientia Horticulturae* **2025**, 347, 114180. <https://doi.org/10.1016/j.scienta.2025.114180>.
2. Wróblewski, M*.; Krajewski, K.; **Gocek, N.**; Żabka, A.; Polit, J. T. *Acorus calamus* L. Essential Oil Induces Oxidative Stress and DNA Replication Disruptions in Root Meristem Cells of Two Fabaceae and Two Brassicaceae Species. *IJMS* **2025**, 26 (10), 4715. <https://doi.org/10.3390/ijms26104715>.
3. Wróblewski, M.; **Gocek, N.**; Żabka, A.; Polit, J. T.* Mitotic Disruption and Cytoskeletal Alterations Induced by *Acorus calamus* Essential Oil: Implications for Bioherbicidal Potential. *IJMS* **2025**, 26 (18), 8933. <https://doi.org/10.3390/ijms26188933>.
4. Wróblewski, M.; **Gocek-Szczurtek, N.**; Krajewski, K.; Żabka, A.; Polit, J. T.* Ultrastructural, Signaling, and Epigenetic Responses to *Acorus calamus* L. Essential Oil in Root Meristems of Fabaceae and Brassicaceae Seedlings. *The Plant Journal* **2025**, manuskrypt nieopublikowany

polegał na:

- współuczestnictwie w pracach technicznych związanych z przygotowaniem prac eksperymentalnych oraz doradztwie w zakresie przygotowania figur i paneli zdjęciowych ich układu, czytelności i spójności wizualnej.

* - autor korespondujący


.....
Podpis



PODPIS ZAUFANY

JUSTYNA MARIA
GÓRSKA

23.09.2025 14:53:27 (GMT+2)

Dokument podpisany elektronicznie
podpisem zaufanym

Gdańsk, 23.09.2025

dr inż. Justyna Górską
Katedra Technologii Leków i Biochemii
Wydział Chemiczny
Politechnika Gdańska

Oświadczenie współautora publikacji

Oświadczam, że mój udział w publikacji:

1. Szczepblewski P.*; Wróblewski M.; Borzyszkowska-Bukowska J.; Bairamova T.; **Górska J.**; Laskowski T.; Samulewicz A.; Kosno M.; Sobiech Ł.; Polit J.T.; Kukula-Koch W. The role of centrifugal partition chromatography in the removal of β -asarone from *Acorus calamus* essential oil. *Scientific Reports* **2022**, 12, 22217, <https://doi.org/10.1038/s41598-022-26726-6>

polegał na:

- współuczestnictwie w przeprowadzaniu eksperymentów

* - autor korespondujący



Raport weryfikacji

Wynik
Wynik przetwarzania
Komunikat błędu

Skrót pliku z podpisem
Nazwa pliku z podpisem
Identyfikator weryfikacji

WAŻNY/PRAWIDŁOWY
Zakończono
Podpis elektroniczny nie został złożony za pomocą kwalifikowanego
urządzenia do składania podpisu (QSCD)
844f31a2fbb8c2c56bb82b581a90af408da1f0cf
udział J. Górską (1).pdf
98D04F33256BD0DECE1C43F5ADE3ACB6854C8106

Lista podpisów

Status weryfikacji podpisu
Data i czas wykonania podpisu
Data i czas weryfikacji podpisu
Format podpisu
Poziom podpisu
Certyfikat podpisującego

WAŻNY/PRAWIDŁOWY
2025-09-23 14:53:27 GMT+02:00
2025-09-25 19:34:52 GMT+02:00
PAdES
BES (Podpis w postaci podstawowej)
kwalifikowany

Osoba podpisująca (profil zaufany ePUAP)

Imię i nazwisko
JUSTYNA MARIA GÓRSKA

Ścieżka certyfikacji

Certyfikat podpisującego

Walidacja certyfikatu
Status certyfikatu
Wystawiony dla

Wystawiony przez
Ważny od
Ważny do
Data wystawienia CRL/OCSP
Numer seryjny

WAŻNY/PRAWIDŁOWY
WAŻNY
CN=Minister do spraw informatyzacji - pieczęć podpisu zaufanego,
O=Ministerstwo Cyfryzacji, C=PL
CN=Centrum Kwalifikowane EuroCert, O=EuroCert Sp. z o.o., C=PL,
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2025-09-25 19:00:02 GMT+02:00
efd46f5b31b845d08071c5eed94d4eb9d37d

Certyfikat wystawcy

Walidacja certyfikatu
Status certyfikatu
Wystawiony dla
Wystawiony przez
Ważny od
Ważny do
Numer seryjny

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CN=Centrum Kwalifikowane EuroCert, O=EuroCert Sp. z o.o., C=PL,
CN=Narodowe Centrum Certyfikacji, O=Narodowy Bank Polski, C=PL
2017-02-14 13:26:19 GMT+01:00
2028-02-15 00:59:59 GMT+01:00
1a5734b0d472d251e1d37cfe3d796ac117102490

Gdańsk, 23.09.2025

dr inż. Michał Kosno
Katedra Technologii Leków i Biochemii
Wydział Chemiczny
Politechnika Gdańska

Oświadczenie współautora publikacji

Oświadczam, że mój udział w publikacji:

1. Szczepblewski P.*; Wróblewski M.; Borzyszkowska-Bukowska J.; Bairamova T.; Górka J.; Laskowski T.; Samulewicz A.; **Kosno M.**; Sobiech Ł.; Polit J.T.; Kukula-Koch W. The role of centrifugal partition chromatography in the removal of β -asarone from *Acorus calamus* essential oil. *Scientific Reports* **2022**, 12, 22217, <https://doi.org/10.1038/s41598-022-26726-6>

polegał na:

- współuczestnictwie w przeprowadzaniu eksperymentów

* - autor korespondujący



PODPIS ZAUFANY

MICHAŁ
KOSNO

22.09.2025 23:47:52 [GMT+2]
Dokument podpisany elektronicznie
podaniem zaufanym



Raport weryfikacji

Wynik

Wynik przetwarzania
Komunikat błędu

Skrót pliku z podpisem
Nazwa pliku z podpisem
Identyfikator weryfikacji

WAŻNY/PRAWIDŁOWY

Zakończono

Podpis elektroniczny nie został złożony za pomocą kwalifikowanego urządzenia do składania podpisu (QSCD)
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Lista podpisów

Status weryfikacji podpisu
Data i czas wykonania podpisu
Data i czas weryfikacji podpisu
Format podpisu
Poziom podpisu
Certyfikat podpisującego

WAŻNY/PRAWIDŁOWY

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2025-09-25 19:34:20 GMT+02:00
PAdES
BES (Podpis w postaci podstawowej)
kwalifikowany

Osoba podpisująca (profil zaufany ePUAP)

Imię i nazwisko
MICHAŁ KOSNO

Ścieżka certyfikacji

Certyfikat podpisującego

Walidacja certyfikatu
Status certyfikatu
Wystawiony dla

WAŻNY/PRAWIDŁOWY

WAŻNY

CN=Minister do spraw informatyzacji - pieczęć podpisu zaufanego,
O=Ministerstwo Cyfryzacji, C=PL
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2027-02-12 10:27:09 GMT+01:00
2025-09-25 19:00:02 GMT+02:00
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Wystawiony przez
Ważny od
Ważny do
Data wystawienia CRL/OCSP
Numer seryjny

Certyfikat wystawcy

Walidacja certyfikatu
Status certyfikatu
Wystawiony dla
Wystawiony przez
Ważny od
Ważny do
Numer seryjny

WAŻNY/PRAWIDŁOWY

WAŻNY

CN=Centrum Kwalifikowane EuroCert, O=EuroCert Sp. z o.o., C=PL,
CN=Narodowe Centrum Certyfikacji, O=Narodowy Bank Polski, C=PL
2017-02-14 13:26:19 GMT+01:00
2028-02-15 00:59:59 GMT+01:00
1a5734b0d472d251e1d37cfe3d796ac117102490



Castelldefels, 28.09.2025

dr Konrad Krajewski
Katedra Cytofizjologii
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

Oświadczenie współautora publikacji

Oświadczam, że mój udział w publikacji:

1. Wróblewski, M*.; **Krajewski, K.**; Gocek, N.; Żabka, A.; Polit, J. T. *Acorus calamus* L. Essential Oil Induces Oxidative Stress and DNA Replication Disruptions in Root Meristem Cells of Two Fabaceae and Two Brassicaceae Species. *IJMS* **2025**, 26 (10), 4715.
<https://doi.org/10.3390/ijms26104715>.

polegał na: współuczestnictwie w wykonywaniu obrazów z mikroskopii konfokalnej

2. Wróblewski, M*.; Gocek-Szczurtek, N.; **Krajewski, K.**; Żabka, A.; Polit, J. T. Ultrastructural, Signaling, and Epigenetic Responses to *Acorus calamus* L. Essential Oil in Root Meristems of Fabaceae and Brassicaceae Seedlings. *The Plant Journal* **2025**, manuskrypt nieopublikowany

polegał na: współuczestnictwie w opracowywaniu immunofluorescencyjnej metody identyfikacji aktywnych kinaz MAP.

* - autor korespondujący



Raport weryfikacji

Wynik

Wynik przetwarzania

Komunikat błędu

Skrót pliku z podpisem

Nazwa pliku z podpisem

Identyfikator weryfikacji

WAŻNY/PRAWIDŁOWY

Zakończono

Podpis elektroniczny nie został złożony za pomocą kwalifikowanego urządzenia do składania podpisu (QSCD)

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udział K. Krajewski - cyfPodpis.pdf

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Lista podpisów

Status weryfikacji podpisu

Data i czas wykonania podpisu

Data i czas weryfikacji podpisu

Format podpisu

Poziom podpisu

Certyfikat podpisującego

WAŻNY/PRAWIDŁOWY

2025-09-28 21:24:21 GMT+02:00

2025-09-29 06:41:41 GMT+02:00

PAdES

BES (Podpis w postaci podstawowej)

kwalifikowany

Osoba podpisująca (profil zaufany ePUAP)

Imię i nazwisko

: KONRAD SZCZEPAN KRAJEWSKI

Ścieżka certyfikacji

Certyfikat podpisującego

Walidacja certyfikatu

Status certyfikatu

Wystawiony dla

Wystawiony przez

Ważny od

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Data wystawienia CRL/OCSP

Numer seryjny

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Certyfikat wystawcy

Walidacja certyfikatu

Status certyfikatu

Wystawiony dla

Wystawiony przez

Ważny od

Ważny do

Numer seryjny

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2017-02-14 13:26:19 GMT+01:00

2028-02-15 00:59:59 GMT+01:00

1a5734b0d472d251e1d37cfe3d796ac117102490

Lublin, 20.09.2025

prof. dr hab. Wirginia Kukuła-Koch
Zakład Farmakognozji z Ogrodem Roślin Leczniczych
Katedra Farmakognozji i Botaniki Farmaceutycznej
Wydział Farmaceutyczny
Uniwersytet Medyczny w Lublinie

Oświadczenie współautora publikacji

Oświadczam, że mój udział w publikacji:

1. Szczepblewski P.*; Wróblewski M.; Borzyszkowska-Bukowska J.; Bairamova T.; Górka J.; Laskowski T.; Samulewicz A.; Kosno M.; Sobiech Ł.; Polit J.T.; **Kukuła-Koch W.** The role of centrifugal partition chromatography in the removal of β -asarone from *Acorus calamus* essential oil. *Scientific Reports* **2022**, 12, 22217, <https://doi.org/10.1038/s41598-022-26726-6>

polegał na:

- współuczestnictwie w opracowaniu koncepcji badań i projektowaniu eksperymentów, analizie wyników, przygotowaniu manuskryptu głównego oraz w opracowaniu figur, przygotowaniu i weryfikacji poprawionej wersji manuskryptu.

* - autor korespondujący

Wirginia Anna
Kukuła-Koch

Elektronicznie podpisany przez
Wirginia Anna Kukuła-Koch
Data: 2025.09.20 17:37:16
+02'00'

.....
Podpis



Raport weryfikacji

Wynik

Wynik przetwarzania
Skrót pliku z podpisem
Nazwa pliku z podpisem
Identyfikator weryfikacji

WAŻNY/PRAWIDŁOWY

Zakończono

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udział W. Kukuła-Koch_sig.pdf
814BAEC2CA6C256ABBFA26DC52397391CAEE6B5D

Lista podpisów

Status weryfikacji podpisu
Data i czas wykonania podpisu
Data i czas weryfikacji podpisu
Format podpisu
Poziom podpisu
Certyfikat podpisującego

WAŻNY/PRAWIDŁOWY

2025-09-20 17:37:16 GMT+02:00
2025-09-20 18:52:13 GMT+02:00
PKCS7
BES (Podpis w postaci podstawowej)
kwalifikowany

Ścieżka certyfikacji

Certyfikat podpisującego

Walidacja certyfikatu
Status certyfikatu
Wystawiony dla

WAŻNY/PRAWIDŁOWY

WAŻNY

surname=Kukuła-Koch, givenName=Wirginia Anna, CN=Wirginia Anna
Kukuła-Koch, O=Uniwersytet Medyczny w Lublinie, C=PL,
CN=COPE SZAFIR - Kwalifikowany, O=Krajowa Izba Rozliczeniowa S.A., C=PL
2023-12-22 08:00:00 GMT+01:00
2025-12-22 08:00:00 GMT+01:00
2025-09-20 17:37:10 GMT+02:00
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Wystawiony przez
Ważny od
Ważny do
Data wystawienia CRL/OCSP
Numer seryjny

Certyfikat wystawcy

Walidacja certyfikatu
Status certyfikatu
Wystawiony dla
Wystawiony przez
Ważny od
Ważny do
Numer seryjny

WAŻNY/PRAWIDŁOWY

WAŻNY

CN=COPE SZAFIR - Kwalifikowany, O=Krajowa Izba Rozliczeniowa S.A., C=PL
CN=Narodowe Centrum Certyfikacji, O=Narodowy Bank Polski, C=PL
2021-12-06 11:36:59 GMT+01:00
2032-12-07 00:59:59 GMT+01:00
062aac8d84b0ddcc

Gdańsk, 23.09.2025

dr hab. inż. Tomasz Laskowski
Katedra Technologii Leków i Biochemii
Wydział Chemiczny
Politechnika Gdańska

Oświadczenie współautora publikacji

Oświadczam, że mój udział w publikacji:

1. Szczepblewski P.*; Wróblewski M.; Borzyszkowska-Bukowska J.; Bairamova T.; Górka J.; **Laskowski T.**; Samulewicz A.; Kosno M.; Sobiech Ł.; Polit J.T.; Kukula-Koch W. The role of centrifugal partition chromatography in the removal of β -asarone from *Acorus calamus* essential oil. *Scientific Reports* **2022**, 12, 22217, <https://doi.org/10.1038/s41598-022-26726-6>

polegał na:

- współuczestnictwie w analizie wyników, przygotowaniu manuskryptu głównego oraz w opracowaniu figur.

* - autor korespondujący





PODPIS ZAUFANY

ALICJA
PIOTROWSKA-NICZYPORUK
22.09.2025 09:32:11 (GMT+2)
Dokument podpisany elektronicznie
podpisem zaufanym

Białystok, 23.09.2025

dr hab. Alicja Piotrowska-Niczyporuk
Katedra Biologii i Ekologii Roślin
Wydział Biologii
Uniwersytet w Białymstoku

Oświadczenie współautora publikacji

Oświadczam, że mój udział w publikacji:

1. Wróblewski, M*.; **Piotrowska-Niczyporuk, A.**; Ciereszko, I.; Gocek, N.; Żabka, A.; Szczepblewski, P.; Sobiech, Ł.; Saja-Garbarz, D.; Polit, J. T. Phytotoxicity and Bioherbicidal Potential of Sweet Flag (*Acorus calamus* L.) Essential Oil on Fabaceae and Brassicaceae Species. *Scientia Horticulturae* **2025**, 347, 114180. <https://doi.org/10.1016/j.scienta.2025.114180>.

polegał na:

- współuczestnictwie w: planowaniu pracy eksperymentalnej (obejmującej analizę stresu oksydacyjnego, systemu antyoksydacyjnego i metabolitów komórkowych), opracowywaniu i dostosowywaniu metodyki badawczej, zapewnieniu materiałów oraz przeprowadzaniu doświadczeń w mojej jednostce badawczej, w której odbywał się pobyt doktoranta,
- uczestnictwie w walidacji uzyskanych wyników,
- wprowadzaniu korekt metodycznych do przygotowywanego manuskryptu,

* - autor korespondujący

.....
Podpis



Raport weryfikacji

Wynik

Wynik przetwarzania

Komunikat błędu

Skrót pliku z podpisem

Nazwa pliku z podpisem

Identyfikator weryfikacji

WAŻNY/PRAWIDŁOWY

Zakończono

Podpis elektroniczny nie został złożony za pomocą kwalifikowanego urządzenia do składania podpisu (QSCD)

d26b4fa7d5092bebf5498fa71893515be6af8d3f

udział A.Piotrowska-Niczyporuk podpis.pdf

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Lista podpisów

Status weryfikacji podpisu

Data i czas wykonania podpisu

Data i czas weryfikacji podpisu

Format podpisu

Poziom podpisu

Certyfikat podpisującego

WAŻNY/PRAWIDŁOWY

2025-09-22 09:32:11 GMT+02:00

2025-09-22 12:53:04 GMT+02:00

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Imię i nazwisko

ALICJA PIOTROWSKA-NICZYPORUK

Ścieżka certyfikacji

Certyfikat podpisującego

Walidacja certyfikatu

Status certyfikatu

Wystawiony dla

Wystawiony przez

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Certyfikat wystawcy

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2017-02-14 13:26:19 GMT+01:00

2028-02-15 00:59:59 GMT+01:00

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Oświadczenie współautora publikacji

Oświadczam, że mój udział w publikacji:

1. Szczepblewski P.*; Wróblewski M.; Borzyszkowska-Bukowska J.; Bairamova T.; Górka J.; Laskowski T.; Samulewicz A.; Kosno M.; Sobiech Ł.; **Polit J.T.**; Kukula-Koch W. The role of centrifugal partition chromatography in the removal of β -asarone from *Acorus calamus* essential oil. *Scientific Reports* **2022**, 12, 22217, <https://doi.org/10.1038/s41598-022-26726-6>

polegał na:

- pozyskaniu materiału badawczego z wskazanego w pracy regionu S3 Polski i przygotowaniu mapy lokalizacji miejsc pochodzenia pobranego ze środowiska materiału badawczego.
- współuczestnictwie w: opracowaniu koncepcji badań, walidacji uzyskanych wyników, pisaniu manuskryptu i wprowadzaniu korekt do przygotowywanego manuskryptu oraz do jego poprawek zgodnych z zaleceniami recenzentów.

Oświadczam, że mój udział w poniżej wymienionych publikacjach:

1. Wróblewski, M*.; Piotrowska-Niczyporuk, A.; Ciereszko, I.; Gocek, N.; Żabka, A.; Szczepblewski, P.; Sobiech, Ł.; Saja-Garbarz, D.; **Polit, J. T.** Phytotoxicity and Bioherbicidal Potential of Sweet Flag (*Acorus calamus* L.) Essential Oil on Fabaceae and Brassicaceae Species. *Scientia Horticulturae* **2025**, 347, 114180. <https://doi.org/10.1016/j.scienta.2025.114180>.
2. Wróblewski, M*.; Krajewski, K.; Gocek, N.; Żabka, A.; **Polit, J. T.** *Acorus calamus* L. Essential Oil Induces Oxidative Stress and DNA Replication Disruptions in Root Meristem Cells of Two Fabaceae and Two Brassicaceae Species. *IJMS* **2025**, 26 (10), 4715. <https://doi.org/10.3390/ijms26104715>.
3. Wróblewski, M.; Gocek, N.; Żabka, A.; **Polit, J. T.*** Mitotic Disruption and Cytoskeletal Alterations Induced by *Acorus calamus* Essential Oil: Implications for Bioherbicidal Potential. *IJMS* **2025**, 26 (18), 8933. <https://doi.org/10.3390/ijms26188933>.
4. Wróblewski, M.; Gocek-Szczurtek, N.; Krajewski, K.; Żabka, A.; **Polit, J. T.*** Ultrastructural, Signaling, and Epigenetic Responses to *Acorus calamus* L. Essential Oil in Root Meristems of Fabaceae and Brassicaceae Seedlings. *The Plant Journal* **2025**, manuskrypt nieopublikowany

polegał na:

- wsparciu merytorycznym w pozyskaniu finansowania badań
- współuczestnictwie w: opracowaniu koncepcji badań, planowaniu pracy eksperymentalnej oraz opracowywaniu i dostosowywaniu metodyki badawczej,
- sprawowaniu nadzoru merytorycznego nad prowadzonymi badaniami,
- uczestnictwie w walidacji uzyskanych wyników,
- wprowadzaniu korekt do przygotowywanego manuskryptu oraz do jego poprawek zgodnych z zaleceniami recenzentów.

* - autor korespondujący



Podpis

Kraków, 22.09.2025

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Zakład Ekofizjologii
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Polskiej Akademii Nauk w Krakowie

Oświadczenie współautora publikacji

Oświadczam, że mój udział w publikacji:

1. Wróblewski, M*.; Piotrowska-Niczyporuk, A.; Ciereszko, I.; Gocek, N.; Żabka, A.; Szczepblewski, P.; Sobiech, Ł.; **Saja-Garbarz, D.**; Polit, J. T. Phytotoxicity and Bioherbicidal Potential of Sweet Flag (*Acorus calamus* L.) Essential Oil on Fabaceae and Brassicaceae Species. *Scientia Horticulturae* **2025**, 347, 114180. <https://doi.org/10.1016/j.scienta.2025.114180>.

polegał na:

- współuczestnictwie w opracowaniu metodyki badań z wykorzystaniem kalorymetrii izotermicznej oraz nadzór nad realizacją tych eksperymentów w mojej jednostce badawczej, w której odbywał się pobyt doktoranta.

* - autor korespondujący



PODPIS ZAUFANY

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SAJA-GARBARZ
28.09.2025 15:22:12 GMT+0200
Dokument podpisany elektronicznie
podpisem zaufanym

.....
Podpis



Raport weryfikacji

Wynik

Wynik przetwarzania

Komunikat błędu

Skrót pliku z podpisem

Nazwa pliku z podpisem

Identyfikator weryfikacji

WAŻNY/PRAWIDŁOWY

Zakończono

Podpis elektroniczny nie został złożony za pomocą kwalifikowanego urządzenia do składania podpisu (QSCD)

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udział D.Saja-Garbarz podpisany.pdf

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Lista podpisów

Status weryfikacji podpisu

Data i czas wykonania podpisu

Data i czas weryfikacji podpisu

Format podpisu

Poziom podpisu

Certyfikat podpisującego

WAŻNY/PRAWIDŁOWY

2025-09-28 15:22:12 GMT+02:00

2025-09-28 16:20:28 GMT+02:00

PAdES

BES (Podpis w postaci podstawowej)

kwalifikowany

Osoba podpisująca (profil zaufany ePUAP)

Imię i nazwisko

: Diana Saja-Garbarz

Ścieżka certyfikacji

Certyfikat podpisującego

Walidacja certyfikatu

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Wystawiony dla

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2017-02-14 13:26:19 GMT+01:00

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Oświadczenie współautora publikacji

Oświadczam, że mój udział w publikacji:

1. Szczepblewski P.*; Wróblewski M.; Borzyszkowska-Bukowska J.; Bairamova T.; Górka J.; Laskowski T.; **Samulewicz A.**; Kosno M.; Sobiech Ł.; Polit J.T.; Kukula-Koch W. The role of centrifugal partition chromatography in the removal of β -asarone from *Acorus calamus* essential oil. *Scientific Reports* **2022**, 12, 22217, <https://doi.org/10.1038/s41598-022-26726-6>

polegał na:

- współuczestnictwie w opracowaniu figury 2.
- przygotowaniu poprawionej wersji manuskryptu

* - autor korespondujący

Anna Samulewicz

Poznań, 23.09.2025

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Oświadczenie współautora publikacji

Oświadczam, że mój udział w publikacji:

1. Szczepblewski P.*; Wróblewski M.; Borzyszkowska-Bukowska J.; Bairamova T.; Górka J.; Laskowski T.; Samulewicz A.; Kosno M.; **Sobiech Ł.**; Polit J.T.; Kukula-Koch W. The role of centrifugal partition chromatography in the removal of β -asarone from *Acorus calamus* essential oil. *Scientific Reports* **2022**, 12, 22217, <https://doi.org/10.1038/s41598-022-26726-6>

polegał na:

- współuczestnictwie w opracowaniu koncepcji badań i projektowaniu eksperymentów,

Oświadczam, że mój udział w publikacji:

1. Wróblewski, M*.; Piotrowska-Niczyporuk, A.; Ciereszko, I.; Gocek, N.; Żabka, A.; Szczepblewski, P.; **Sobiech, Ł.**; Saja-Garbarz, D.; Polit, J. T. Phytotoxicity and Bioherbicidal Potential of Sweet Flag (*Acorus calamus* L.) Essential Oil on Fabaceae and Brassicaceae Species. *Scientia Horticulturae* **2025**, 347, 114180. <https://doi.org/10.1016/j.scienta.2025.114180>.

polegał na:

- merytorycznym wsparciu w wyborze tatarakowego olejku eterycznego, opracowaniu metody emulgowania olejku i dostarczeniu emulgatora

* - autor korespondujący



PODPIS ZAUFANY

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SOBIECH

22.09.2025 12:13:02 [GMT+2]

Dokument podpisany elektronicznie
podpisem zaufanym

.....
Podpis



Raport weryfikacji

Wynik

Wynik przetwarzania

Komunikat błędu

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udział Ł. Sobiech (1).pdf

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Lista podpisów

Status weryfikacji podpisu

Data i czas wykonania podpisu

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WAŻNY/PRAWIDŁOWY

2025-09-22 12:13:02 GMT+02:00

2025-09-22 12:53:36 GMT+02:00

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Osoba podpisująca (profil zaufany ePUAP)

Imię i nazwisko

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Ścieżka certyfikacji

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Gdańsk, 23.09.2025

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Oświadczenie współautora publikacji

Oświadczam, że mój udział w publikacji:

1. **Szczęblewski P. ***; Wróblewski M.; Borzyszkowska-Bukowska J.; Bairamova T.; Górka J.; Laskowski T.; Samulewicz A.; Kosno M.; Sobiech Ł.; Polit J.T.; Kukula-Koch W. The role of centrifugal partition chromatography in the removal of β -asarone from *Acorus calamus* essential oil. *Scientific Reports* **2022**, 12, 22217, <https://doi.org/10.1038/s41598-022-26726-6>

polegał na:

- współuczestnictwie w opracowaniu koncepcji badań i projektowaniu eksperymentów, przeprowadzaniu eksperymentów, analizie wyników, przygotowaniu manuskryptu głównego oraz w opracowaniu figur, przygotowaniu i weryfikacji poprawionej wersji manuskryptu.

Oświadczam, że mój udział w publikacji:

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polegał na: współuczestnictwie w zapewnieniu olejku tatarakowego i badaniach jego składu chemicznego

* - autor korespondujący



mgr Mateusz Wróblewski
Katedra Cytofizjologii
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

Oświadczenie współautora publikacji

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polegał na:

- współuczestnictwie w przeprowadzeniu eksperymentu dotyczącego składu tatarakowego olejku eterycznego stosowanego w pracy doktorskiej, przygotowaniu manuskryptu głównego oraz w opracowaniu figur w tym zakresie, przygotowaniu i weryfikacji poprawionej wersji manuskryptu w tym zakresie.

Oświadczam, że mój udział w poniżej wymienionych publikacjach:

1. **Wróblewski, M.***; Piotrowska-Niczyporuk, A.; Ciereszko, I.; Gocek, N.; Żabka, A.; Szczepblewski, P.; Sobiech, Ł.; Saja-Garbarz, D.; Polit, J. T. Phytotoxicity and Bioherbicidal Potential of Sweet Flag (*Acorus calamus* L.) Essential Oil on Fabaceae and Brassicaceae Species. *Scientia Horticulturae* **2025**, 347, 114180. <https://doi.org/10.1016/j.scienta.2025.114180>.
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3. **Wróblewski, M.**; Gocek, N.; Żabka, A.; Polit, J. T.* Mitotic Disruption and Cytoskeletal Alterations Induced by *Acorus calamus* Essential Oil: Implications for Bioherbicidal Potential. *IJMS* **2025**, 26 (18), 8933. <https://doi.org/10.3390/ijms26188933>.
4. **Wróblewski, M.**; Gocek-Szczurtek, N.; Krajewski, K.; Żabka, A.; Polit, J. T.* Ultrastructural, Signaling, and Epigenetic Responses to *Acorus calamus* L. Essential Oil in Root Meristems of Fabaceae and Brassicaceae Seedlings. *The Plant Journal* **2025**, manuskrypt nieopublikowany

polegał na:

- pozyskaniu finansowania badań i administrowaniu projektem,
- współuczestnictwie w: opracowaniu koncepcji badań, planowaniu pracy eksperymentalnej, opracowaniu i dostosowaniu metodyki badawczej, walidacji uzyskanych wyników,
- przeprowadzeniu eksperymentów przy wsparciu pomocy technicznej i opiekunów poszczególnych zadań w macierzystej jednostce badawczej oraz w jednostkach zewnętrznych,
- zarządzaniu danymi badawczymi
- przygotowaniu manuskryptu,
- przygotowaniu szaty graficznej przy wsparciu w zakresie walorów estetycznych - układu, czytelności i spójności,
- współudziale w korekcie manuskryptu zgodnej z zaleceniami recenzentów.

* - autor korespondujący



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Oświadczenie współautora publikacji

Oświadczam, że mój udział w poniższych publikacjach:

1. Wróblewski, M*.; Piotrowska-Niczyporuk, A.; Ciereszko, I.; Gocek, N.; **Żabka, A.**; Szczeblewski, P.; Sobiech, Ł.; Saja-Garbarz, D.; Polit, J. T. Phytotoxicity and Bioherbicidal Potential of Sweet Flag (*Acorus calamus* L.) Essential Oil on Fabaceae and Brassicaceae Species. *Scientia Horticulturae* **2025**, 347, 114180. <https://doi.org/10.1016/j.scienta.2025.114180>.

polegał na współudziale w zapewnieniu materiałów badawczych

2. Wróblewski, M*.; Krajewski, K.; Gocek, N.; **Żabka, A.**; Polit, J. T. *Acorus calamus* L. Essential Oil Induces Oxidative Stress and DNA Replication Disruptions in Root Meristem Cells of Two Fabaceae and Two Brassicaceae Species. *IJMS* **2025**, 26 (10), 4715. <https://doi.org/10.3390/ijms26104715>.

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3. Wróblewski, M.; Gocek, N.; **Żabka, A.**; Polit, J. T.* Mitotic Disruption and Cytoskeletal Alterations Induced by *Acorus calamus* Essential Oil: Implications for Bioherbicidal Potential. *IJMS* **2025**, 26 (18), 8933. <https://doi.org/10.3390/ijms26188933>.

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* - autor korespondujący

Aneta Żabka
.....
Podpis