WYDZIAŁ BIOLOGII
i OCHRONY ŚRODOWISKA
Uniwersytet Łódzki

Stacjonarne Studia Doktoranckie
Mikrobiologii, Biotechnologii
i Biologii Eksperymentalnej

**Izabela Kołodziejczyk**

# Egzogenna melatonina jako czynnik modyfikujący metabolizm kiełkujących nasion kukurydzy (*Zea mays* L.)

Exogenous melatonin as a factor modifying the metabolism of germinating maize seeds (*Zea mays* L.)

Praca doktorska

wykonana w Katedrze Ekofizjologii Roślin
Instytutu Biologii Eksperymentalnej
Uniwersytetu Łódzkiego

pod kierunkiem
prof. dr hab. Małgorzaty M. Posmyk

Łódź, 2021

**Spis treści**



**Załączniki**

1. Kopie publikacji wchodzących w skład rozprawy doktorskiej wraz z oświadczeniami współautorów (**Zał. 1-4**).
2. Manuskrypt wchodzących w skład rozprawy doktorskiej z oświadczeniem współautorów i potwierdzeniem z *Int. J. Mol. Sci.* o przyjęciu go do recenzji (**Zał. 5**).
3. Wydruk raportu *Web.Sci*. z dn. 19.05.2021 r. na temat danych bibliometrycznych publikacji wchodzących w skład rozprawy doktorskiej (**Zał. 6**).

## 1. Źródła finansowania badań prowadzonych w ramach pracy doktorskiej

Badania finansowane w latach 2011-2015,

Grant NCN N N310 111940

„Proteomiczna analiza zmian profili białkowych w osiach zarodkowych nasion kondycjonowanych z melatoniną eksponowanych na stres chłodu podczas kiełkowania" –

kierownik: prof. dr hab. Małgorzata M. Posmyk.

## 2. Współpraca

dr hab. Andrzej Kaźmierczak, prof. UŁ

Katedra Cytofizjologii
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

dr hab. Katarzyna Dzitko, prof. UŁ,

Katedra Mikrobiologii Molekularnej
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

dr Rafał Szewczyk

Katedra Mikrobiologii Przemysłowej i Biotechnologii
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

**3. Wykaz elementów wchodzących w skład mieszanej rozprawy doktorskiej**

**PUBLIKACJE**

**Kołodziejczyk I., Posmyk M.M. (2016)** Melatonin - a new plant biostimulator? *Journal of Elementology* 21(4): 1187-1197 DOI: 10.5601/jelem.2015.20.3.1012.

**IF 0.641**$^{2016}$, **0.781**$^{V}$; **14** cytowań* (**40**$^{A}$ pkt-y MEiN)

**Kołodziejczyk I., Bałabusta M., Szewczyk R., Posmyk M.M. (2015)** The levels of melatonin and its metabolites in conditioned corn (*Zea mays* L.) and cucumber (*Cucumis sativus* L.) seeds during storage. *Acta Physiologiae Plantarum* 37(6): 105 DOI :10.1007/s11738-015-1850-7.

**IF 1.563**$^{2015}$, **2.078**$^{V}$; **17** cytowań* (**70**$^{A}$ pkt-y MEiN)

**Kołodziejczyk I., Dzitko K., Szewczyk R., Posmyk M.M. (2016a)** Exogenous melatonin expediently modifies proteome of maize (*Zea mays* L.) embryo during seed germination. *Acta Physiologiae Plantarum* 38: 146 DOI: 10.1007/s11738-016-2166-y

**IF 1.364**$^{2016}$, **2.078**$^{V}$; **12** cytowań* (**70**$^{A}$ pkt-y MEiN)

**Kołodziejczyk I., Dzitko K., Szewczyk R., Posmyk M.M. (2016b)** Exogenous melatonin improves corn (*Zea mays* L.) embryo proteome in seeds subjected to chilling stress. *Journal of Plant Physiology* 193: 77-56 DOI:10.1016/j.jplph.2016.01.012.

**IF 3.121**$^{2016}$, **3.615**$^{V}$; **30** cytowań* (**100**$^{A}$ pkt-y MEiN)

**MANUSKRYPT**

**Kołodziejczyk I., Kaźmierczak A., Posmyk M.M. (2021)** Melatonin application modifies antioxidant defence and induces endoreplication in maize seeds exposed to chilling stres. *International Journal of Molecular Science*

Manuskrypt przyjęty do oceny dn. 25.05.2021 r. **IF 4.556**$^{2019}$, **4.653**$^{V}$ (**140**$^{A}$ pkt-y MEiN)

Na dysertację doktorską składają się:

- 4 opublikowane prace o łącznym **IF** z dnia publikacji **6,689** oraz aktualnym pięcioletnim **IF 8,556**$^{V}$, łączną sumą punktów wg. listy MEiN **280** - prace te były cytowane **73** razy*;
- 1 manuskrypt przyjęty do recenzji w *International Journal of Molecular Science* (IF 4.556$^{2019}$, 4.653$^{V}$; 140$^{A}$ punkty MEiN).

*Dane bibliometryczne z dn. 19.05.2021 r. wg. *Web.Sci* (Zał. 6).

## 4. Omówienie celu naukowego i uzyskanych wyników

### 4.1. Wprowadzenie

Istotnym kierunkiem badań z zakresu biologii i uprawy roślin jest poznanie mechanizmów odporności i tolerancji odporności istotnych gospodarczo roślin względem zmieniających się, często niekorzystnych warunków środowiska. Wrażliwość roślin na stresy jest różna w zależności od stadium rozwojowego. Szczególnie newralgicznym okresem jest moment kiełkowania nasion i wschody siewek. Z tego powodu jakość nasion, ich wigor i żywotność, jak również odpowiednie przygotowanie do wysiewu, jest kluczem dla prawidłowego rozwoju młodej siewki i jej przetrwania w potencjalnie niekorzystnych warunkach środowiska.

W celu polepszania jakości nasion od lat z powodzeniem stosuje się zabiegi kondycjonowania/primingu, takie jak: hydro-, osmo- lub matri-kondycjonowanie [Lutts i wsp., 2016] Priming nasion opiera się na kontrolowanym uwadnianiu ich suchych tkanek określoną eksperymentalnie ilością wody, która pozwala na uruchomienie wstępnych faz kiełkowania, lecz nie dopuszcza do wzrostu zarodka i przebicia okrywy nasiennej przez korzeń zarodkowy. Taka ograniczona imbibicja nasion umożliwia ich powtórną desykację (ostatni etap kondycjonowania), a następnie ewentualne przechowywanie do momentu wysiewu [Hasanuzzaman i Fotopoullos, 2019]. Odpowiednio dobrany dla gatunku i prawidłowo przeprowadzony zabieg kondycjonowania nasion polepsza parametry kiełkowania m.in.: wydajności, tempo i energię kiełkowania. Ponadto powoduje, że nasiona kiełkują równomiernie nawet w warunkach suboptymalnych np. w szerszym zakresie temperatur, ograniczonym przez zasolenie dostępie wody, czasowej hypoksji itp. [Lutts i wsp., 2016]. Przekłada się to również na równomierne wschody silniejszych, tolerujących stresy siewek.

Poszczególne typy primingu mogą być uzupełnione aplikacją bioaktywnych substancji stymulujących lub/i ochronnych takich jak: regulatory wzrostu roślin, hormony, antyoksydanty, fungicydy, antybiotyki itp. Takie chemiczne uzupełnienie kondycjonowania nasion z założenia powinno potęgować pozytywne efekty zabiegu i nie przysparzać problemów toksykologicznych, więc aplikowane substancje winny być naturalne, a co najmniej biodegradowalne. Tylko poszukiwanie sposobów poszerzenia

tolerancji na stresy bez dodatkowej chemizacji środowiska, otwiera szeroko perspektywę ekologicznych upraw i zdrowych produktów spożywczych pochodzenia roślinnego – w trend ten wpisuje się prezentowana dysertacja.

Jako obiekt badań niniejszej pracy doktorskiej wybrano nasiona (ziarna) kukurydzy cukrowej (*Zea mays* L. var. Ambrozja). Ta jednoliścienna roślina jest szeroko stosowana w żywieniu człowieka. Jej ważną cechą jest to, że nie indukuje glutenozależnych alergii pokarmowych, więc kukurydziane produkty spożywcze są nie tylko smaczne ale i bezpieczne. Zielone części kukurydzy są cenną paszą dla zwierząt gospodarskich, zaś z włókien produkuje się ekologiczne biodegradowalne materiały na opakowania i torby.

Warunki klimatyczne obszaru Polski bywają niewystarczające dla satysfakcjonującej uprawy nawet aklimatyzowanych odmian kukurydzy. Krótki okres wegetacji , a zwłaszcza niskie wiosenne temperatury w okresie kiełkowania, są stresujące i ograniczające dla pochodzącego z Ameryki Południowej gatunku [Ranum i wsp., 2014; Soare i wsp., 2018]. Dlatego każde niskonakładowe polepszenie kiełkowania i wschodów w warunkach wiosennych chłodów, a w efekcie wydłużenie okresu wegetacji i poprawę plonu, który da wymierne korzyści ekonomiczne – jest pożądane. Zgodnie z takim założeniem, w prezentowanej pracy doktorskiej eksperymentalnie dobrano optymalną dla nasion kukurydzy technikę kondycjonowania, którą wzmacniano biostymulującym działaniem melatoniny.

Melatonina (MEL; *N*-acetylo-5-metoksytryptamina), początkowo znana była jako neurohormon zwierzęcy. W efekcie licznych badań dowiedziono, iż jest ona wysoce konserwatywną cząsteczką, obecną w znacznie oddalonych ewolucyjnie organizmach. Endogenna MEL (fitomelatonina) została odkryta i opisana u wielu gatunków roślin. Jej zawartość jest różna i waha się od kilku pg do kilkuset ng w gramie świeżej masy, w zależności od gatunku, odmiany, a nawet organu z którego pobrano materiał do analiz [Hattori i wsp., 1995]. Poziom fitomelatoniny w tkankach jest zależny również od etapu rozwoju i stanu fizjologicznego rośliny i może być związany z warunkami w jakich roślina egzystuje. Stwierdzono, że szczególnie intensywnie MEL jest syntetyzowana w niesprzyjających warunkach środowiska, a rośliny z wyższą jej zawartością lepiej tolerują stresy [Zhang i wsp., 2015; Kołodziejczyk i Posmyk, 2016; Arnao i Hernandez-Ruiz 2021; Tripathi i wsp. 2021].

Fenomen ten wiązano z faktem iż MEL jest wysoce efektywnym uniwersalnym antyoksydantem, niwelującym zarówno reaktywne formy tlenu (RFT) jak i azotu (RFA), co w połączeniu z niewielkimi rozmiarami i amfifilnością (doskonale penetruje wszystkie kompartmenty komórkowe), czyni ją niezwykle skuteczną w ochronie przed wtórnym stresem oksydacyjnym [Kołodziejczyk i Posmyk, 2016]. Co ciekawe, produkty powstające z MEL podczas redukcji wolnych rodników tlenowych i nie rodnikowych RFT takie jak m.in.: hydroksy-melatonina, cykliczna melatonina, czy N(1)-acetyl-N(2)-formyl-5-methoksykinuramina (AFMK) - zachowują właściwości przeciwutleniające. Tworzy więc MEL specyficzną kaskadę antyoksydantów co generuje dużo wyższy potencjał detoksykujący niż ten obserwowany u powszechnie znanych nieenzymatycznych przeciwutleniaczy [Arnao i Hernandez-Ruiz, 2019]. Opisywano także różny, pozytywny wpływ w MEL na działanie enzymów antyoksydacyjnych [Bałabusta i wsp., 2016].

Te własności niewątpliwie sankcjonują obecność MEL w mitochondriach i chloroplastach [Tripathi i wsp., 2021]. Mimo, iż organella te wykształciły wiele mechanizmów ochronnych, MEL wydaje się także być cząsteczką zapewniającą integralność strukturalną i funkcjonalną ich błon [Arnao i Hernandez-Ruiz 2021]. Ponadto, indolamina ta chroni chlorofil przed degradacją co zapewnia utrzymanie chloroplastów w stanie poprawnego funkcjonowania [Szafrańska i wsp., 2017]. i przyczynia się pośrednio do spowolnienia procesów starzenia. Wspomaga więc MEL nieprzerwane prowadzanie najważniejszych procesów życiowych rośliny: fotosyntezy i oddychania komórkowego.
Ponieważ MEL jest analogiem strukturalnym IAA (kwas indolilo-octowy) przypisuje się jej własności auksynopodobne, zwłaszcza, że indukuje rozwój korzeni i moduluje ich architekturę [Arnao i Hernandez-Ruiz, 2017]. Jednak najintensywniej stymuluje MEL wegetatywny wzrost nadziemnych części roślin, co jest widoczne zwłaszcza w warunkach suboptymalnych. Ponieważ zauważono, że pozytywne efekty działania MEL uwidaczniają się zwykle w warunkach różnych stresów (np. temperaturowych, solnego, osmotycznego, suszy, działania metali ciężkich) [Arnao i Hernandez-Ruiz, 2021] wydaje się, że może ona poprawiać sprawność strategii obronnych roślin lub specyficznie akcelerować ich odporność/tolerancję na stresy [Kołodziejczyk i Posmyk, 2016].

Naturalną konsekwencją tych przemyśleń był plan suplementacji nasion kukurydzy odpowiednim, eksperymentalnie ustalonym stężeniem egzogennej MEL dla podwyższenia ich wartość siewnej i zwiększenia prawdopodobieństwa przetrwania wyrosłych z nich

siewek warunków chłodu. Efekty działania MEL podawanej do nasion w drodze ich przedsiewnego hydrokondycjonowania, na kiełkowanie kukurydzy w warunkach stresu chłodu i po jego ustąpieniu, zbadano na poziomie fizjologicznym, biochemicznym, cytofizjologicznym i molekularnym (proteomicznym).

### 4.2. Cel pracy i hipoteza badawcza

Celem pracy było udokumentowanie korzystnego wpływu MEL na metabolizm kiełkujących nasion kukurydzy cukrowej (*Zea mays* L. var. Ambrozja) i zbadanie jakie procesy indukuje/stymuluje MEL w roślinie poszerzając jej tolerancję na stres chłodu i wzmagając proces regeneracji po jego ustąpieniu.

Pierwotna hipoteza badawcza zakładała, że: (1) przedsiewne kondycjonowanie będzie efektywną metodą aplikacji MEL do nasion, (2) znacznie podniesiony poziom MEL w nasionach ograniczy negatywne działanie chłodu podczas kiełkowania, poprzez ograniczenie wtórnego stresu oksydacyjnego, (3) celowym jest prześledzenie potencjalnych zmian w proteomie zarodków kiełkujących nasion dla ustalenia procesów metabolicznych modulowanych przez kondycjonowanie i przez egzogenną MEL.

Wyniki badań proteomicznych sprowokowały do postawienia kolejnej hipotezy wtórnej zakładającej, że (4) ilościowe zmiany białek generowane pod wpływem MEL mogą być rezultatem powielenia materiału genetycznego w strefach bazalnych zarodków przez endoreplikacje.

Nakreślony cel pracy osiągnięto realizując wymienione poniżej cele szczegółowe (etapy).

- Charakterystyka materiału siewnego:
  - testy kiełkowania w gamie temperatur, ustalenie warunków stresu chłodu;
  - kinetyka imbibicji - ustalenie inicjalnej i finalnej/granicznej wilgotności nasion;
  - dobór metody i warunków kondycjonowania dla nasion kukurydzy;
  - dobór optymalnego stężenia MEL aplikowanej do nasion
  - testy porównawcze pokroju/wielkości zarodków.

  Działania te pozwoliły na (i) ustalenie warunków eksperymentu - czasu trwania i intensywność stresu - oraz (ii) wybór wariantów nasion do porównania.
- Badanie zawartości MEL i jej metabolitów w materiale roślinnym:

- oznaczenie poziomu endogennej MEL w nasionach kukurydzy;
- szacowanie efektywności i trwałości wybranej metody podawania egzogennej MEL, poprzez oznaczenie poziomu tej indoloaminy w nasionach zaraz po zabiegach kondycjonowania i co miesiąc przez rok ich przechowywania;
- identyfikacja pochodnych/metabolitów MEL.

- Obserwacje efektów kondycjonowania i MEL w kontekście wtórnego stresu oksydacyjnego generowanego chłodem:
  - szacowanie uszkodzeń oksydacyjnych;
  - analizy aktywności enzymów antyoksydacyjnych i detoksykujących.
- Analizy proteomiczne białek izolowanych z osi zarodkowych nasion kiełkujących w warunkach optymalnych i stresu chłodu:
  - charakterystyka zmian generowanych kondycjonowaniem;
  - charakterystyka zmian generowanych przez MEL.
- Identyfikacja zmian zachodzących za pośrednictwem MEL na poziomie cyklu komórkowego w strefach bazalnych osi zarodkowych kiełkujących nasion.

### 4.3. Metodyka badań

**Materiał roślinny**

Materiał roślinny stanowiły nasiona kukurydzy cukrowej (*Zea mays* L., var. Ambrozja) zakupione w firmie nasienniczej TORSEED (Toruń, Polska). Przed rozpoczęciem eksperymentów były one przechowywane w szczelnie zamkniętych pojemnikach, w ciemności, w temperaturze pokojowej.

**Testy kiełkowania**

Testy kiełkowania w warunkach laboratoryjnych, z wykorzystaniem komór fitotronowych (gama temperatur 1-25 $^{o}$C; podłoża osmotycznie czynne w zakresie 0-2 -MPa – roztwory PEG-8000) prowadzono według zasad ISTA (ang. *International Seed Testing Association*).

**Ustalenie warunków i dobór optymalnego typu primingu**

Wykonano i testowano dwa typy primingu: osmo- i hydrokondycjonowanie; uzupełniane o aplikację MEL w różnych stężeniach (zakres 25-500 µM). Rezultaty zabiegów kondycjonowania były monitorowane testami fizjologicznymi.

**Analiza pokroju zarodków**

Wypreparowane z wybranych wariantów nasion zarodki były fotografowane, a ich powierzchnia mierzona przy pomocy oprogramowania ImageJ.

**Monitoring zawartości MEL i jej pochodnych w nasionach**

Poziom MEL i jej analoga strukturalnego – IAA oznaczono metodą wysokosprawnej chromatografii cieczowej z detekcją elektrochemiczną (HPLC-EC).

Analizy ilościowe i jakościowe, dotyczące MEL i jej metabolitów w wybranych wariantach nasion przechowywanych przez rok prowadzono przy użyciu techniki HPLC-MS/MS tj. wysokosprawnej chromatografii cieczowej ze spektrometrem masowym.

**Badanie markerów uszkodzeń oksydacyjnych**

W określonych momentach eksperymentu i wybranych wariantach nasion, oceniano stopień peroksydacji lipidów (test TBARS) oraz utleniania białek (test OxiProt).

**Analizy aktywności enzymów antyoksydacyjnych i detoksykujących**

Aktywność enzymów: dysmutazy ponadtlenkowej (SOD), katalazy (CAT), peroksydazy askorbinianowej (APX), peroksydazy glutationowej (GSH-PX), peroksydaz niespecyficznych (GPX), reduktazy glutationowej (GSSG-R) i S-transferazy glutationowej (GST), oznaczano - w określonych momentach eksperymentu i wybranych wariantach nasion - metodami biochemicznymi, analizując spektrofotometrycznie kinetykę ich reakcji.

**Analizy proteomiczne**

W celu rozdzielenia oczyszczonych i zagęszczonych ekstraktów białkowych pochodzących z zarodków wybranych wariantów nasion kiełkujących w warunkach optymalnych i w stresie chłodu, wykorzystane zostały techniki 2D tj. ogniskowanie izoelektryczne (IEF) i elektroforeza w warunkach denaturujących (SDS-PAGE). Do obrazowania map

proteomicznych, analiz graficznych i statystycznych oraz typowania spotów użyto oprogramowania PDQuest. Pozwoliło to na wyselekcjonowanie, charakterystycznych dla danych wariantów, plam białkowych oraz określenie podstawowej puli proteomowej. Wytypowane spoty białkowe były trawione i poddane analizie HPLC MS/MS. Każde białko zostało zidentyfikowane, nazwane, przypisane do rodziny i sklasyfikowane wg funkcji w oparciu o bazę danych Mascot / przy użyciu narzędzi MASKOT, NCBI i DELTA-BLAST.

**Metody cytofizjologiczne**

Przekroje zarodków z wybranych wariantów nasion zostały wybarwione fluorochromem DAPI (4'-6-diamidyno-2-fenylindol). Barwnik ten interkaluje w miejsca DNA bogate w pary zasad AT. Posłużyło to wizualizacji mikroskopowej w świetle UV ilości materiału genetycznego w jądrach komórkowych. Obserwacje były dokumentowane fotograficznie. Analizom została poddana strefa bazalna zarodka, czyli ta, w której wraz z jego wzrostem zachodzą zmiany związane z dyferencjacją komórek. Niesłusznym byłoby badać cały zarodek, ponieważ w merystemach korzeniowym i zawiązku pędu, zachodzi w trakcie wzrostu tylko proliferacja i nie determinuje ona kierunku rozwoju namnażanych komórek. Na podstawie pomiarów densytometrycznych wykonanych za pomocą oprogramowania ScnImage, poprzez odniesienie intensywności fluorescencji materiału genetycznego w poszczególnych jądrach do podstawowej ilości DNA, utworzono histogramy rozkładu zawartości materiału genetycznego i otrzymano procentowy udział jąder poliploidalnych.

**Analizy statystyczne**

Dane analizowano za pomocą oprogramowania STATISTICA v.10.0. W zależności od potrzeby stosowano jedno- lub dwukierunkową analizę wariancji (ANOVA), a następnie test *post-hoc* Duncana, aby znaleźć istotne różnice, które określano w każdym eksperymencie przy $p < 0,01$.

### 4.4. Omówienie wyników

Badania wstępne – m.in. testy fizjologiczne kiełkowania i inne określające żywotność i wigor nietraktowanych nasion (NT) – pozwoliły na ustalenie odpowiednich warunków eksperymentu. Optimum temperaturowe kiełkowania nasion kukurydzy określono na 25 $^{o}$C – w tej temperaturze w ciągu 24 h obserwowano 96% skiełkowań. Suboptymale temperatury zmieniały głównie tempo kiełkowania i stopniowo ilość skiełkowań. Dopiero w temperaturze 5 $^{o}$C obserwowano zdecydowane zahamowanie tego procesu – przez 2 tygodnie imbibicji w chłodzie skiełkowało 11% nasion ale większość tak inkubowanych pozostawała żywa. Niższa temperatura (1 $^{o}$C) szybko ograniczała ich żywotność (wyników testu tetrazolinowego nie prezentowano). Na tej podstawie wyznaczono warunki eksperymentu: 25 $^{o}$C / 24 h jako optimum dla kiełkowania nasion kukurydzy (C – kontrola), 5 $^{o}$C / 14 d jako stres chłodu (S) i 5 $^{o}$C / 14 + 25 $^{o}$C / 24 h jako warunki wystarczając dla zaobserwowania procesów regeneracji (R) po ustąpieniu stresu [Kołodziejczyk i wsp., 2021].

Testowano dwa typy primingu: hydrokondycjonowanie i osmokondycjonowanie. Oznaczono wilgotność inicjalną nasion (8.84% ±0.23) i końcową/graniczną (38.01% ±1.12) do której można je uwodnić dla bezpiecznego/prawidłowego przeprowadzenia zabiegu przedsiewnego kondycjonowania.

Niestety okazało się, że ziarna kukurydzy nie powinny pozostawać zbyt długo w stanie podwyższonej wilgotności, czego wymaga osmokondycjonowania (25 $^{o}$C / -1,5 MPa / 3 d). Długotrwała (liczona w dniach) inkubacja nasion o dużej zawartości skrobi w podwyższonej wilgotności powoduje utratę ich zdolności do bezpiecznego i prawidłowego wtórnego suszenia – zaburza więc ostatni etap kondycjonowania. Ma to prawdopodobnie związek z aktywacją alfa-amylaz i nieodwracalną mobilizacją zasobów poprzez rozkład skrobi. W konsekwencji osmokondycjonowane nasiona kukurydzy kiełkowały gorzej niż nasiona NT (dane nieprezentowane).

W przypadku hydrokondycjonowania istnieją dwa sposoby kontrolowania wilgotności nasion podczas imbibicji: (1) ograniczając ilość wody dodawanej bezpośrednio do nasion lub (2) kontrolując czas moczenia nasion. W pierwszym przypadku należy zwrócić uwagę na jednorodny dostęp nasion do określonej ilości wody obliczonej dla danej ich porcji (wagi), dlatego hydrokondycjonowanie powinno być prowadzone w obracających się

pojemnikach/naczyniach, a woda dodawana sukcesywnie. Niestety, kanciaste i suche ziarna kukurydzy ulegały uszkodzeniom mechanicznym podczas rotacji naczynia. W związku z powyższym zastosowano statyczne ich namaczanie w nieograniczonej ilość wody, przy czym na podstawie badania kinetyki imbibicji określono czas namaczania niezbędny dla uzyskania tzw. finalnej/granicznej wilgotności nasion. Wykazano, że 3 h moczenia nasion kukurydzy w temperaturze 25 $^{o}$C wystarcza do uzyskania 36,8% ±2 ich wilgotności. Po wykonaniu zabiegu hydrokondycjonowania (25 $^{o}$C / 3 h namaczania nasion w natlenionej wodzie destylowanej lub wodnych roztworach MEL) nasiona suszono do wilgotności inicjalnej na otwartych tacach, w temperaturze pokojowej, przez kolejne 3 dni i takie przechowywano, a następnie wykorzystywano do testów.

Udokumentowano, że dobrze dobrana technika hydrokondycjonowania zastosowana do nasion kukurydzy zapewnia zadowalające wyniki zarówno pod względem kiełkowania nasion, jak i wzrostu osi zarodkowych w warunkach optymalnych i stresu chłodu [Kołodziejczyk i wsp., 2021]. Efekty hydrokondycjonowania nasion zostały dodatkowo wzmocnione przez zastosowanie naturalnego biostymulatora - MEL. Testowano jej stężenia w zakresie 25-500 µM. Na podstawie ww. testów fizjologicznych do doświadczeń biochemicznych, proteomicznych i cytofizjologicznych wybrano następujące warianty nasion: nietraktowane (NT – nasiona kontrolne), hyrokondycjonowane wodą (H – wtórna kontrola pozwalająca określić efekty samego zabiegu kondycjonowania, dla odróżnienia od efektów MEL) i hydrokondycjonowane w dwóch roztworach wodnych MEL 50 i 500 µM (akronimy odpowiednio: HMel 50 i HMel 500). Dawka MEL 50 µM została określona jako optymalna – tak traktowane nasiona osiągały najlepsze wyniki w testach fizjologicznych; natomiast dawkę 500 µM traktowano jako wariant, w którym obserwowano efekty przedawkowania (patrz kinetyka kiełkowanie w gamie temperatur i wzrost osi zarodkowych) [Kołodziejczyk i wsp., 2021].

Badane ziarna kukurydzy (NT, H) zawierały 30-35 ng endogennej MEL na gram świeżej masy. Zabieg hydroprimingu z MEL okazał się być niezwykle efektywnym w podwyższeniu jej zawartości w nasionach – do wartości ~1 µg/$g_{FW}$ w wariancie HMel 50 i aż 20-21 µg/$g_{FW}$ w wariancie HMel 500 [Kołodziejczyk i wsp., 2021].

Przy okazji ww. oznaczeń HPLC-EC zbadano również zmiany w zawartości analoga strukturalnego MEL – kwasu indolilooctowego (IAA – auksyna). Zastosowanie 50 µM MEL podczas hydropriming nasion kukurydzy nie zmieniło w nich poziomu IAA, tylko 500 µM

MEL zmniejszyła jego ilość do 43% kontroli NT. Należy jednak zaznaczyć, że hydropriming (sama metoda zaprawiania nasion) zwiększył poziom IAA w nasionach o około 35%, stąd korelacja ujemna względem NT mogła być widoczna tylko przy wyższym stężeniu MEL.
W kontekście dostępnej literatury, wydaje się, że wpływ MEL na IAA zależy od zastosowanej ilości MEL, jednak ważna jest również metoda jej podawania i docelowy organ rośliny. Ponieważ jednak różne badania wykazują zarówno dodatnią, jak i ujemną korelację między MEL i IAA, problem ten należałoby jeszcze rozpatrywać w szerszej sieci zależności modulujących ekspresję czynników transkrypcyjnych związanych z auksynami.

Niestety, ze względu na brak monoklonalnych przeciwciał przeciwko MEL i występowanie w komórkach roślinnych szeregu jej analogów strukturalnych (auksyny), nie udało się zwizualizować, gdzie w nasionach lokuje się MEL. Przeprowadzono zaś z sukcesem eksperyment dotyczący fluktuacji zawartości MEL podczas rocznego przechowywania wszystkich wariantów nasion [Kołodziejczyk i wsp., 2015].
Okazało się, że nasiona nietraktowane egzogenną MEL (tj. NT i H) pomimo stałych warunki przechowywania (szczelnie zamknięte pojemniki, ciemność, temperatura pokojowa) wykazywały sezonowe zmiany stężenia tej indoloaminy, ze znacznym ok. 3-krotnym wzrostem jej poziomu w miesiącach zimowych. Te sezonowe zmiany – zauważone po raz pierwszy w KER przy badaniach nasion jednoliściennej kukurydzy i dwuliściennego ogórka [Kołodziejczyk i wsp., 2015] – sugerują istnienie nieregulowanej przez czynniki zewnętrzne, swego rodzaju „pamięci biochemicznej" pór roku, tj. zegara biologicznego, który działa jako kalendarz biochemiczny (prawdopodobnie rozwinięty ewolucyjnie w umiarkowanym, zmiennym klimacie) regulowany przez fitomelatoninę.

W okresie rocznego przechowywania nasion kukurydzy w obu wariantach znacznie wzbogaconych egzogenną MEL (HMel 50 i HMel 500) obserwowano stopniowy spadek tej indoloaminy [Kołodziejczyk i wsp., 2015]. Tempo jej utylizowania było charakterystyczne dla gatunku. Dzięki jakościowej analizie HPLC-MS/MS u kukurydzy zidentyfikowano trzy główne pochodne MEL: beta-hydroksymelatoninę, 3-(etano-1-ol)-5-metoksyindol i cykliczną melatoninę. Wszystkie ww. pochodne są produktami utleniania MEL. To sugeruje, że podczas przechowywania nasion MEL ulegając utlenianiu w stosunkowo suchych tkankach nasion (prawdopodobnie bez aktywnych enzymów antyoksydacyjnych), chroni je przed stresem oksydacyjnym zapobiegając uszkodzeniom generowanym w trakcie długotrwałego przechowywania.

Należy wyjaśnić rozbieżności jakie wystąpiły w wartościach przy różnych oznaczeniach MEL. W przypadku nasion NT i H (12-14 ng/gFW w publiacji Kołodziejczyk i wsp., 2015; 30-35 ng/gFW w manuskrypcie Kołodziejczyk i wsp., 2021) mogły one wyniknąć (i) z powodu różnych roczników nasion poddawanych analizie oraz (ii) wykrytej sezonowej fluktuacji fitomelatoniny. Natomiast, w przypadku nasion HMel50 i HMel500 zawyżone w publikacji Kołodziejczyk i wsp., 2015 wyniki oznaczeń wykonanych zaraz po kondycjonowaniu wyniknęły prawdopodobnie z większej czułości zastosowanego tu detektora MS/MS.

W prezentowanych badaniach wykazano, że MEL aplikowana do nasion modyfikuje działanie podstawowych enzymów antyoksydacyjnych i detoksykujących, redukując wtórny stres oksydacyjny i uszkodzenia generowane chłodem [Kołodziejczyk i wsp., 2021]. Zimno jest stresem specyficznym - ze względu na spowolnienie reakcji biochemicznych i konserwujący efekt niskich temperatur, czasami trudno jest zaobserwować negatywne skutki podczas trwania chłodu. Dlatego tak ważne są obserwacje i analizy wykonywane po przeniesieniu roślin do optymalnych warunków - uszkodzenia spowodowane przez zimno są lepiej widoczne w okresie regeneracji w wyższej temperaturze.

Przedłużający się stres chłodu nasila peroksydację lipidów i białek. Wykazano, że osie zarodkowe z nasion przedsiewnie traktowanych MEL kumulują znacznie mniej TBARS (produktów utleniania lipidów) niż NT, zarówno podczas stresu, jak i po jego ustąpieniu. Ponadto analiza stopnia karbonylacji białek wykazała, że nasiona z podwyższoną zawartością MEL nie wykazywały (HMel 500) lub nieznacznie (HMel 50) wzrostu utleniania białek w porównaniu z jego poziomem w optymalnych warunkach temperaturowych. Taka ochrona przed chłodem błon białkowo-lipidowych i działających w nich enzymów jest z pewnością powodem skutecznej regeneracji i szybszego wzrostu osi zarodkowych z nasion hydrokondycjonowanych z MEL, co udokumentowano fotograficznie i pomiarem ich powierzchni.

Opisane wyżej efekty mają związek z bezpośrednimi właściwościami przeciwutleniającymi MEL, jak również z jej wpływem na aktywność enzymów antyoksydacyjnych [Kołodziejczyk i wsp., 2021]. Wykazano, że chociaż SOD z zarodków nasion kukurydzy wydaje się być wrażliwa na chłód 5 $^{o}$C – bo jej aktywność znacznie spadła w niskiej temperaturze i nie regenerowała się po ustąpieniu stresu do poziomu

osiąganego przy 25 $^{o}$C przez rośliny inkubowane przez cały czas w optymalnych warunkach – to jednak, w warunkach stresu chłodu i po nim, aktywność SOD była 2-2,5 razy większa w nasionach traktowanych MEL niż w nasionach NT i H. Szczególnie intensywnie stymulowane przez MEL były peroksydazy: askorbinianowa (APX) i glutationowa (GSH-PX) oraz S-transferaza glutationowa (GST); i chociaż wspomniane enzymy glutationo-zależne (GSH-PX i GST) wydają się być również wrażliwe na niską temperaturę 5 °C (w chłodzie działały słabo), to po przeniesieniu kiełkujących nasion HMel 50 i HMel 500 do 25 °C szybko odzyskały niezwykle wydajną funkcjonalność. Natomiast reduktaza glutationu (GSSG-R) w nasionach hydrokondycjonowanych z MEL była dwukrotnie bardziej aktywna zarówno w warunkach stresu chłodu, jak i po jego ustąpieniu, w porównaniu z nasionami NT i H. Pomaga to z pewnością w utrzymaniu korzystnej puli zredukowanego glutationu w komórkach.

Istotną częścią badań prezentowanej dysertacji doktorskiej były ilościowe i jakościowe analizy proteomów ekstrahowanych z zarodków wszystkich badanych wariantów nasion kiełkujących w warunkach optymalnych i stresu chłodu [Kołodziejczyk i wsp., 2016a; 2016b]. Dzięki nim zostały określone białka, których ekspresja jest indukowana przez MEL oraz, których biosynteza jest znacznie zwiększona w nasionach przedsiewnie traktowanych MEL.

Wiadomo, że już sam zabieg hydrokondycjonowania modyfikuje proteom nasion. Porównując uzyskane mapy białkowe zarodków nasion kiełkujących w 25 $^{o}$C stwierdzono, że przy stałej puli 129 białek typowych dla wszystkich wariantów nasion, zabieg hydroprimingu generował dodatkowe 14 białek, a MEL kolejne 14 wspólnych dla obu aplikowanych dawek (50 i 500 µM) i 23 charakterystycznych dla HMel 50 oraz 3 charakterystyczne dla HMel 500. Aplikacja MEL spowodowała znaczący, w stosunku do nasion NT i H, wzrost zawartości enzymów metabolizmu energetycznego, białek zaangażowanych w tzw. „plastyczność proteomu", tj. tych które usprawniają biosyntezę, składanie i transport nowych białek do miejsc ich funkcjonowania, a także białek detoksykujących, obronnych i anty-stresowych – mimo że analizowano proteomy z nasion kiełkujących w warunkach optymalnych 25 $^{o}$C. Białka te są bardzo często uważane za markery jakości nasion – szczególnie HSP, LEA, TRX i podobne białka stresowe. Rozpoznając je i wskazując jako powstające po aplikacji MEL do nasion, częściowo wyjaśniono dlaczego wyrosłe z takich nasion siewki są silniejsze i sprawniej adaptują się

do suboptymalnych warunków środowiska – są z góry lepiej przygotowane do obrony przed potencjalnymi stresami (działanie prewencyjne MEL) [Kołodziejczyk i wsp., 2016a].

Pomimo, iż metabolizm zwalnia i biosynteza białek jest silnie ograniczana przez niską temperaturę 5 $^{o}$C, pozytywne modyfikacje w proteomie zarodków izolowanych z nasion H, a zwłaszcza HMEL 50, zaobserwowano również podczas ich kiełkowania w stresie chłodu [Kołodziejczyk i wsp., 2016b]. Tym razem mapy białkowe zarodków nasion kiełkujących w 5 $^{o}$C prezentowały, stałą pulę 78 białek typowych dla wszystkich wariantów nasion, zabieg hydroprimingu generował w warunach stresu dodatkowe 3 białka, 5 białek było wspólnych dla nasion H i obu wariantów HMEL; 6 typowych dla HMEL; 2 dla HMel 50 i 5 dla HMel 500. Zmiany indukowane przez MEL wiązały się z mobilizacją rezerw, intensyfikacją oddychania i metabolizmu energetycznego. Tego typu modyfikacje, ze względu na zwiększone potrzeby energetyczne w warunkach stresu, są kluczowe dla przetrwania go. Ponadto w osiach zarodkowych izolowanych z nasion traktowanych MEL pojawiało się znacznie więcej białek stresowych, obronnych i detoksykujących, kilka specyficznych białek, które mogą pełnić rolę selekcjonujących ligaz E3 w roślinnym ubikwityno-zależnym systemie degradacji protein, a także szereg dodatkowych białek o charakterze opiekuńczym (ang. *chaperons*). Plastyczność proteomu (biosynteza, postranslacyjne modyfikacje, „dojrzewanie" i inkorporacja białek w komórce) ale także selektywna degradacja warunkująca „obrót białek", ma istotne znaczenie dla przetrwania niekorzystnych warunków – kiedy-to generowane są uszkodzenia starych i perturbacje w metabolizmie nowo powstających protein – oraz umożliwiają efektywną regenerację po ustąpieniu stresu.

Wszystkie wskazane pozytywne modyfikacje proteomu zarodków wywoływane przez hydrokondycjonowane, a zwłaszcza hydrokondycjonowanie połączone z aplikacją MEL do nasion kukurydzy, wskazują ścieżki metaboliczne odpowiedzialne za fenomen lepszej tolerancji stresu i skuteczniejszej regeneracji po jego ustąpieniu u roślin rozwijających się z tak traktowanych nasion (działanie interwencyjne MEL) [Kołodziejczyk i wsp., 2016b].

Ponieważ w badaniach proteomicznych zaobserwowano nie tylko jakościowy, ale i ilościowy wzrost puli białek osi embrionalnych izolowanych z nasion traktowanych MEL, pojawiły się pytania: jak to możliwe, a zwłaszcza jak jest to możliwe w warunkach stresu chłodu, gdy metabolizm zwalnia?

Ponieważ skład białkowy i wielkość jego puli jest następstwem dostępności matryc DNA, fenomen ilościowego wzrostu puli białek w osiach embrionalnych izolowanych z nasion traktowanych MEL i kiełkujących w stresie chłodu skojarzono z ewentualną poliploidalnością. Powszechnie wiadomo, że w komórkach poliploidalnych obserwuje się bardziej efektywny i produktywny metabolizm. Jeżeli komórki wchodzą w endocykle, dochodzi do dodatkowych rund syntezy DNA i w efekcie końcowym zwielokrotnienia ostatecznego produktu multiplikowanych genów – białek funkcyjnych. Z kolei, zwiększeniu zawartości materiału genetycznego w jądrze komórkowym towarzyszy zwiększenie objętości całej komórki, co opisuje tzw. współczynnik jądrowo-plazmatyczny. Komórki przybierające na objętości akcelerują wzrost. Ponadto, dostępna literatura wskazuje, że mutanty roślinne z supresją syntezy, transportu i sygnalizacji auksyn, wykazują szybkie przełączanie mitozy na endocykl oraz osiągają podwyższony poziom poliploidii [De Veyler i wsp., 2011]. Te znane fakty, zbieżne z naszymi wynikami – zwiększoną syntezą białek, obniżeniem poziomu IAA i szybszym wzrostem osi zarodkowych pod wpływem podawanej do nasion MEL – były przesłankami do sprawdzenia, czy MEL może także indukować endoreplikację w rosnących osiach zarodkowych kukurydzy [Kołodziejczyk i wsp., 2021].

Pomiary mikrocytfotometryczne jąder wybarwionych fluorochromem DAPI i ich transformacja cyfrowa, pozwoliły stwierdzić, że egzogenna MEL (zwłaszcza w dawce 50 µM) stymuluje pojawienie się endocykli w osiach zarodkowych nasion kukurydzy już podczas kiełkowania w temperaturze optymalnej 25 $^{o}$C (C). Osie zarodkowe izolowane z wariantów nasion NT i H dały obraz niezróżnicowanych tkanek, w których zachodzi proliferacja, a to skutkuje powolnym, stałym wzrostem zarodka.

Histogramy otrzymane dla nasion kiełkujących w temperaturze 5 $^{o}$C (S) wykazały, że proces proliferacji został zakłócony przez chłód. W wariantach nasion NT i H komórki zarodków najczęściej zatrzymane były w fazie G1, a stosunkowo niewiele komórek znajdowało się w innych fazach cyklu. Największa liczba endoreplikujących komórek w warunkach stresu pojawiła się w zarodkach nasion przedsiewnie traktowanych MEL – około 40% jąder komórkowych w HMel 50 i 20% w HMel 500.

Pomiary wykonane po stresie – w czasie regeneracji (R) w przypadku osi zarodkowych pochodzących z nasion wariantów NT i H ujawniły pogłębienie stanu obserwowanego podczas inkubacji w 5 $^{o}$C. Prawie 100% jąder pozostawało w fazie G1, nie

odnotowano powrotu do proliferacji, a nawet zaobserwowano uszkodzenia materiału genetycznego – pofragmentowanie. Tłumaczyło to zły stan tych roślin. Z kolei w osiach zarodkowych nasion hydrokondycjonowanych z MEL ponownie zidentyfikowano wiele jąder poliploidalnych, szczególne dużo (około 40%) w wariancie HMel 50 – co wyjaśniało ich szybki wzrost podczas regeneracji.

W prezentowanej pracy po raz pierwszy wskazano na związek aplikowania egzogennej MEL do nasion kukurydzy z występowaniem endocykli w strefie bazalnej zarodków w trakcie i po stresie chłodu – w kontekście uruchomienia kolejnej strategii dla zwiększenia przeżywalności roślin.

Wiadomo, że endoreplikacja występuje jako naturalny sposób kompensowania przez rośliny ewolucyjnych strat w małym genomie. Jest to więc strategia mająca na celu uzyskanie większej ilości produktów końcowych, których rośliny potrzebują w różnych warunkach środowiskowych z którymi muszą się zmierzyć [De Veyler i wsp., 2011]. Dobrze byłoby więc mieć w dyspozycji narzędzie, które pozwalałoby taką strategię indukować i kontrolować dla polepszenia jakości i ilości plonów. Naszym zdaniem MEL jest dobrym do tego kandydatem, jednak dalsze badania w tym względzie są konieczne.

Konkludując, podjęte w niniejszej pracy doktorskiej badania wyjaśniają na poziomie fizjologicznym, biochemicznym, cytologicznym i proteomicznym fenomen pozytywnego działania egzogennej MEL na kiełkowanie nasion oraz wzrost zarodków. Sugerują one również, dlaczego rośliny bogate w endogenną MEL wydają się być bardziej odporne na stresy i na odwrót, dlaczego stresy generują zwiększoną syntezę tej indoloaminy u niektórych roślin. Prezentowane wyniki uzasadniają ponadto poprawność sklasyfikowania MEL jako obiecującego naturalnego, ekologicznego, fitobiostymulatora, który może być stosowany w roli czynnika prewencyjnego – przygotowującego na stres zanim zaistnieje, jak i interwencyjnego – wspomagającego przezwyciężenie/tolerowanie działającego stresu.

## 4.5. Wnioski

Wyniki badań zawartych w niniejszej rozprawie doktorskiej upoważniają do sformułowania następujących wniosków:

1. Dobrze dobrana technika hydrokondycjonowania zastosowana do nasion kukurydzy zapewnia zadowalające wyniki zarówno pod względem kiełkowania nasion, jak i wzrostu osi zarodkowych w warunkach optymalnych i stresu chłodu. Jednak pozytywne efekty hydrokondycjonowania są zdecydowanie wzmacniane przez zastosowanie naturalnego biostymulatora – MEL, przy czym odpowiednio dobrana dawka jest kluczem do sukcesu.
2. Hydrokondycjonowanie jest wysoce efektywną metodą aplikowania egzogennej MEL do nasion kukurydzy. Zawartość tej indoloaminy wzrasta w nich proporcjonalnie do zastosowanego stężenia MEL. Efekt utrzymuje się co najmniej przez rok, a stopniowe utlenianie MEL w tym czasie może chronić suche tkanki nasion przed stresem oksydacyjnym i zapobiegać uszkodzeniom generowanym podczas długotrwałego przechowywania.
3. Odkryto nową nieopisywaną dotychczas funkcję MEL jako regulatora w biochemicznym kalendarzu nasion. Sezonowe fluktuacje fitomelatoniny (endogenna MEL) ze znacznym wzrostem zimą dają roślinie informacje o niekorzystnym dla kiełkowania okresie.
4. MEL aplikowana do nasion pozytywnie modyfikuje działanie podstawowych enzymów antyoksydacyjnych i detoksykujących (SOD, APX, GSH-PX, GSSG-R i GST) redukując wtórny stres oksydacyjny i uszkodzenia (utlenianie białek i lipidów) generowane podczas kiełkowania w chłodzie i po jego ustąpieniu, warunkując lepszą regeneracją roślin. Przy czym, nie jest zmieniana pula białek enzymatycznych tylko ich aktywność.
5. Opisano prewencyjne i interwencyjne ścieżki działania MEL na poziomie molekularnym. Badania proteomiczne wskazały, że już podczas kiełkowania w warunkach optymalnych (25 $^{o}$C) aplikowana do nasion MEL wzbogaca pulę białek osi zarodkowych o dodatkowe enzymy metabolizmu energetycznego, białka warunkujące plastyczności proteomu oraz białka obronne, antystresowe i detoksykujące. Proteiny należące do tych grup funkcyjnych to w większości markery jakości nasion. Analogiczne zmiany indukowane przez MEL w proteomie zarodków nasion

kiełkujących w stresie chłodu (5 $^{o}$C) – wiążące się z mobilizacją rezerw, intensyfikacją oddychania i metabolizmu energetycznego, plastycznością proteomu w tym sprawnym „obrotem białek”, a więc i detoksykacją komórek – są szczególnie istotne dla przetrwania stresu i sprawnej regeneracji uszkodzeń po jego ustąpieniu.

6. Kolejną strategią, którą indukuje MEL podawana do nasion w celu przezwyciężenia stresu chłodu jest poliploidyzacja przez endoreplikację którą obserwowano w komórkach bazalnej części zarodków. Komórki poliploidalne zdecydowanie lepiej dostosowują się do zmian środowiskowych, w tym niekorzystnych.

Biorąc pod uwagę osiągnięcia prezentowanej pracy doktorskiej wydaje się, że MEL może stanowić niedrogi, bezpieczny i łatwy w aplikacji biostymulator roślinny, który mógłby być stosowany powszechnie w uprawach ekologicznych.

## Bibliografia:

*Prace cytowane w tekście i zaznaczone na szary kolor to publikacje wchodzące w skład rozprawy doktorskiej podane na str. 5 i umieszczone w załącznikach 1-5.*


**Arnao M.B, Hernandez-Ruiz J. (2021)** Melatonin against environmental plant stressors: a review. *Curr Protein Pept Sci.* DOI: 10.2174/1389203721999210101235422.

**Arnao M.B, Hernandez-Ruiz J. (2019)** Melatonin: A New Plant Hormone and/or a Plant Master Regulator? *Trends in Plant Science* 24(1): 38-48. DOI: 10.1016/j.tplants.2018.10.010

**Arnao M.B, Hernandez-Ruiz J. (2017)** Melatonin and its relationship to plant hormones. *Annals of botany* 00: 1-13. DOI: 10.1093/aob/mcx114

**Bałabusta, M.; Szafrańska, K.; Posmyk, M.M. (2016)** Exogenous melatonin improves antioxidant defence in cucumber seeds (*Cucumis sativus* L.) germinated under chilling stress. *Front. Plant Sci.* 7: 575. DOI: 10.3389/fpls.2016.00575

**De Veyler, L.; Larkin, J.C.; Schnittger, A. (2011)** Molecular control and function of endoreplication in development and physiology. *Trends Plant Sci.* 16(11): 624-34. DOI: 10.1016/j.tplants.2011.07.001

**Hasanuzzaman M., Fotopoulos V. (2019)** Priming and Pretreatment of Seeds and Seedlings. *Springer Nature Singapore* ISBN 978-981-13-8624-4. DOI: 10.1007/978-981-13-8625-1

**Hattori, A., Migitaka, H., Iigo, M., Itoh, M., Yamamoto, K., Ohtani-Kaneko, R., Hara, M., Suzuki, T., Reiter, R.J. (1995)**. Identification of melatonin in plants and its effect on plasma melatonin levels and binding to melatonin re-ceptors in vertebrates. *Biochem. Mol. Biol. Int.* 35: 627-634.

**Lutts S., Benincasa P., Wojtyla L., Kubala Sz., Pace R., Lechowska K., Quinet M., Garnczarska M. (2016)** Seed priming: new comprehensive approaches for an old empirical technique. W: Susana Araujo and Alma Balestrazzi, New Challenges in Seed Biology - Basic and Translational Research Driving Seed Technology, *InTech* 1-49. DOI: 10.5772/64420

**Ranum, P.; Pe˜na-Rosas, J.P.; Nieves Garcia-Casal, M. (2014)** Global maize production, utilization, and consumption. *Ann. N.Y. Acad. Sci.* 1312: 105–112. DOI: 10.1111/nyas.12396

**Soare, E.; Chiurciu, I.A.; Balan, A.V.; David L. (2018)** World market research on maize. *Sciendo* 216-222. DOI: 10.2478/alife-2018-0032

**Szafrańska K., Reiter R.J., Posmyk M.M. (2017)** Melatonin Improves the Photosynthetic Apparatus in Pea Leaves Stressed by Paraquat via Chlorophyll Breakdown Regulation and Its Accelerated de novo Synthesis. *Front. Plant Sci.* 8: 878. DOI: 10.3389/fpls.2017.00878

**Tripathi G.T., Javed Z., Mishra M., Fasake V., Dashora K. (2021)** Phytomelatonin in stress management in agriculture. *Heliyon* 7: e06150. DOI: 10.1016/j.heliyon.2021.e06150

**Zhang N., Sun Q., Zhang H., Cao Y., Weeda S., Ren S., Guo Y.D. (2015)** Roles of melatonin in abiotic stress resistance in plants. *J Exp Bot*, 66(3): 647–656 10.1093/jxd/eru336.

## 5. Streszczenie w języku polskim

Rozprawa doktorska dotyczy biostymulujących właściwości melatoniny (MEL, N-acetylo-5-metoksytryptamina) aplikowanej do nasion kukurydzy podczas hydrokondycjonowania. Do badań fizjologicznych, biochemicznych, cytologicznych i molekularnych, użyto nasion nietraktowanych – kontrolnych (NT), hydrokondycjonowanych wodą (H) i wodnymi roztworami MEL o stężeniach 50 i 500 µM (odpowiednio HMel 50 i HMel 500). Wykazano, że aplikowanie egzogennej MEL do nasion poprawiało ich kiełkowanie w temperaturach suboptymalnych i stymulowało wzrost osi zarodkowych w warunkach stresu chłodu (5 $^{o}$C) i po jego ustąpieniu – podczas regeneracji. Do oceny skuteczności kondycjonowania jako metody suplementacji nasion MEL oraz do określenia zawartości MEL i jej potencjalnych metabolitów w nasionach podczas rocznego ich przechowywania zastosowano ilościowe i jakościowe analizy HPLC-MS/MS. Po raz pierwszy, w nasionach NT i H udokumentowano sezonowe wahania stężeń endogennej MEL – zaobserwowano istotny wzrost tej indoloaminy w miesiącach zimowych. Na tej podstawie zasugerowano, że endogenna MEL w nasionach może odgrywać kluczową rolę w sezonowych, niezależnie od warunków środowiskowych, rytmach. Metoda hydrokondycjonowania jest efektywną dla aplikacji MEL do nasion. Poziom tej indoloaminy wzrósł znacznie i proporcjonalnie do zastosowanego stężenia, w nasionach kukurydzy HMel 50 i HMel 500. Zwrócono uwagę, że MEL podczas rocznego przechowywania nasion była stopniowo utleniana, przez co prawdopodobnie chroniła suche tkanki nasion przed stresem oksydacyjnym. Właściwości przeciwutleniające MEL potwierdził niski poziom uszkodzeń oksydacyjnych białek oraz mniejsza ilość produktów peroksydacji lipidów w osiach zarodkowych nasion HMel 50 i HMel 500 eksponowanych na działanie niskiej temperatury (5 $^{o}$C) podczas kiełkowania. Było to skorelowane ze stymulującym wpływem MEL na podstawowe enzymy antyoksydacyjne: SOD, peroksydazy APX i GSH-PX oraz na GST – enzym detoksykujący. W celu zidentyfikowania zmian w proteomie generowanych przez stosowane metody primingu: H, HMel 50 i HMel 500 – ekstrakty białkowe z zarodków nasion kiełkujących w warunkach optymalnych (25 $^{o}$C) i w stresie chłodu (5 $^{o}$C) rozdzielono metodą 2D-PAGE. Następnie uzyskane mapy proteomów porównano (statystycznie i graficznie) za pomocą oprogramowania PDQuest, a charakterystyczne plamy białek przeanalizowano jakościowo technikami spektrometrii mas i zidentyfikowano w bazach danych białek Mascot. Badania

pomogły zidentyfikować białka związane z hydroprimingiem i po raz pierwszy takie, które ulegały ekspresji tylko w obecności MEL. Badania potwierdziły, że odpowiednio dobrany przedsiewny zabieg aplikowania MEL do nasion, poprzez modyfikację proteomu zarodka, skutecznie przygotowuje roślinę *a priori* do niekorzystnych warunków środowiskowych. W zarodkach nasion suplementowanych MEL, nawet w optymalnych warunkach, syntetyzowane były dodatkowe białka o działaniu przeciwutleniającym, odtruwającym i przeciwstresowym oraz enzymy intensyfikujące metabolizm energetyczny komórek. Pozytywne efekty kondycjonowania nasion były szczególnie widoczne podczas kiełkowania w suboptymalnych warunkach. Analogiczne zmiany indukowane przez MEL w proteomie zarodków nasion kiełkujących w warunkach stresu chłodu (5 $^{o}$C) – związane z intensyfikacją oddychania i metabolizmu energetycznego, plastycznością proteomów i wydajnym „obrotem białek", a co za tym idzie również detoksykacją komórek – są szczególnie istotne dla przetrwanie stresu i skutecznej regeneracji uszkodzeń po jego ustąpieniu. Po raz pierwszy udokumentowano, że MEL stymuluje również strategie obronne przed stresem na poziomie cytofizjologicznym, indukując endoreplikację w komórkach bazalnej strefy osi zarodkowych. Podsumowując, MEL aplikowana do nasion kukurydzy poprawia ich jakość, działa biostymulująco i optymalizuje proces kiełkowania zwłaszcza w warunkach stresu chłodu.

**Słowa kluczowe:** biostymulacja, endoreplikacja, enzymy antyoksydacyjne, hydrokondycjonowanie, kiełkowanie, kukurydza, melatonina, metabolity melatoniny, nasiona, peroksydacja lipidów, proteomika, stres chłodu, stres oksydacyjny, utlenianie białek, zaprawianie nasion, *Zea mays*.

## 6. Streszczenie w języku angielskim

The PhD dissertation concerns biostimulating properties of melatonin (MEL, N-acetyl-5-methoxytryptamine) applied to the maize seeds by hydroconditioning. For the tests at the physiological, biochemical cytological and molecular levels, control, non-treated seeds (NT), hydroconditioned with water (H) and with aqueous MEL solutions at 50 and 500 µM concentrations (HMel 50 and HMel 500, respectively) were used. It was demonstrated that the application of exogenous MEL to seeds improved their germination under suboptimal temperatures and stimulated the growth of embryonic axes under chilling stress conditions (5 $^{o}$C) and after stress removal, during regeneration. The HPLC-MS/MS quantitative and qualitative analyses were used to evaluate conditioning efficacy as a method for MEL seed supplementation and to determine the content of MEL and of its potential metabolites in the seeds during 1 year following the conditioning. For the first time, in the NT and H seeds, seasonal fluctuations of endogenous MEL concentration were noted and significant increase in this indoleamine in the winter month was observed. This suggests that in seeds endogenous MEL could play a crucial role in seasonal rhythms independently of environmental conditions. The NT seeds and those conditioned with water (H) contained small amount of endogenous MEL. However, the level of this indoleamine increased markedly in maize seeds primed with exogenous MEL and it was correlated with the concentration of MEL applied. It was noted that MEL was metabolized during seed storage by its gradual oxidation, thus it protects dry seeds against oxidative stress. The antioxidant properties of MEL was also confirmed by low level of protein oxidative damage and smaller quantity of lipid peroxidation products in embryonic axes of HMel 50 and HMel 500 seeds exposed to chilling temperature 5 $^{o}$C during germination. It was correlated with the stimulatory effects of MEL on antioxidant enzymes: SOD, peroxidases: APX and GSH-PX and on GST, a detoxifying enzyme. To identify modifications in proteome of maize seeds caused by all applied conditioning techniques (H, HMel 50, HMel 500), protein extracts of germinated seed embryos in optimal temperature (25 $^{o}$C) and under chilling stress (5 $^{o}$C) were separated by 2D-PAGE. Next, obtained maps of proteomes were compared (statistically and graphically) using PDQuest software, and characteristic spots of proteins were analysed qualitatively by mass-spectrometric techniques and identified in the Mascot protein databases. Research

helped to identify hydropriming-associated proteins and for the first time those which were expressed only in the presence of MEL. Study confirmed that suitably selected pre-sowing treatment with MEL, by embryo proteome modification, *a priori* effectively prepares plants to adverse environmental conditions. In melatonin treated seeds during the initial state of embryos growth, even under optimal conditions, additional antioxidative, detoxifying, anti-stresses proteins and those accelerating energy metabolism were synthesized. Positive effects of seed priming were particularly apparent during germination under suboptimal conditions. Analogous changes induced by MEL in the proteome of seed embryos germinating under cold stress (5 $^{o}$C) – related to the intensification of respiration and energy metabolism, the proteom plasticity and efficient "protein turnover" and thus also the cells detoxification – are particularly important for the survival of stress and efficient regeneration of damage after it has subsided. Moreover it was showed, for the first time, that MEL induced defence strategies against stress at the cytophisiological level, inducing endoreplication in embryonic axes cells. To sum up, MEL applied to corn seeds improves their quality, has a biostimulating effect and optimizes the germination process, especially under cold stress.

**Keywords:** antioxidant enzymes; biostimulation; chilling stress; corn; endoreplication; germination, hydroconditioning, lipid peroxidation, maize, melatonin; melatonin metabolites, oxidative stress, protein oxidation, proteomics, seeds, seed priming; *Zea mays*

## 7. Dorobek naukowy

## 7.1. Spis publikacji niewchodzących w skład rozprawy doktorskiej

*Publikacje popularyzujące naukę:*

**Frasiński S., Kołodziejczyk I. (2014)** Fitoplazmy – bakterie, które przekształcają rośliny w zombi; *Ekonatura* 11(132): 8-9.

**Kołodziejczyk I. (2014)** Typy śmierci komórek roślinnych; *Ekonatura* 12(133): 15-17.

**Kołodziejczyk I., Kopczewski T., Frasiński S. (2015)** Ekologiczna i elektryczna modernizacja szklarni; *Ekonatura* 1(134) str. 18.

**Kołodziejczyk I., Kopczewski T., Frasiński S.** (2015) Jak zabijają rośliny?; *Ekonatura* 2(135) str. 12-13.

**Kopczewski T., Kołodziejczyk I., Frasiński S.** (2015) Alkaloidy – trucizny i leki pochodzące z roślin; Ekonatura 3(136): 10-11.

**Kołodziejczyk I., Kopczewski T., Frasiński S. (2015)** Endoreplikacja u roślin – komórkowa „wygrana na loterii"; *Ekonatura* 4(137): 13-14.

**Frasiński S., Kopczewski T., Kołodziejczyk I. (2015)** Rośliny i metale ciężkie – z gleby na talerz; *Ekonatura* 8(141): 5-6.

**Frasiński S., Kołodziejczyk I., Kopczewski T. (2015)** Co gryzie kapustę, czyli o grzybach *Alternaria*; *Ekonatura* 10(143): 13.

**Kopczewski T., Frasiński S, Kołodziejczyk I. (2015)** W jaki sposób rośliny porozumiewają się ze sobą i z otoczeniem, czyli o lotnych związkach organicznych; *Ekonatura* 11(144): 12.

## 7.2. Doniesienia konferencyjne

## 7.2.1. Wystąpienia ustne

*Konferencje międzynarodowe:*

- Posmyk M.M.**, Kołodziejczyk I**., Rakowiec M., **(2015)** Melatonin as seed biostimulator - physiological and proteomical aspects; Melatonina jako biostymulator nasion – aspekt fizjologiczny i proteomiczny; (**oral presentation**); 2nd International Conference: Biostimulators in modern plant cultivation (25–26 II 2015r. Warsaw/Poland) Book of Abstract pp. **100** (Streszczenia str. **100**)
- **Kołodziejczyk I**., Posmyk M.M. **(2015)** W jaki sposób poprawić wartość siewną nasion kukurydzy? - Melatonina jako fitobiostymulator. Prezentacja ustna KI na II Seminarium Naukowe „Zielone Idee 21 Wieku" (15 X 2015r. Poznań) Materiały konferencjne (sesja referatowa) str. 87
- **Kołodziejczyk I**., Kaźmierczak A., Posmyk M.M. **(2015)** Czy egzogenna melatonina może stymulować endoreplikację w nasionach kukurydzy? **Prezentacja ustna KI** na II Poznanskie Sympozjum Młodych Naukowców nt „Nowe oblicze nauk przyrodniczych" (14 XI 2015r. Poznań) Abstrakty (referat) str. 36

### 7.2.2. Plakaty

*Konferencje międzynarodowe:*

- Posmyk M.M., **Kołodziejczyk I**., Dzitko K., Szewczyk R. **(2015)** Hydropriming supplemented with melatonin modifies corn (*Zea mays* L.) embryo proteome during seed germination under optimal temperature conditions. 7th Conference of Polish Society for Experimental Plant Biology and the Intercollegiate Faculty of Biotechnology UG & MUG (8-11 IX 2015r, Gdansk/Poland) Abstract Book pp. 75
- **Kołodziejczyk I**., Dzitko K., Szewczyk R., Posmyk M.M. **(2015)** Hydropriming supplemented with melatonin modifies corn (*Zea mays* L.) embryo proteome during seed germination under chilling stress conditions. 7th Conference of Polish Society for Experimental Plant Biology and the Intercollegiate Faculty of Biotechnology UG & MUG (8-11 IX 2015r, Gdansk/Poland) Abstract Book pp. 161
- **Kołodziejczyk I**., Kaźmierczak A., Posmyk M.M. **(2015)** The exogenous melatonin involvement in stimulating endocycles occurence in corn seeds. VII International Conference of Biotechnology Students and XVII National Academic Seminar of Biotechnology Students (20-22 XI 2015r, Poznan/Poland) Book of Abstracts pp.96
- **Kołodziejczyk I**., Posmyk M.M. **(2017**) Alternative application of melatonin as phytobiostimulator; 24th Young Researcher Fellow Meeting (8-10 II 2017 Châtenay-Malabry/France) Abstract PC-045
- **Kołodziejczyk I**., Posmyk M.M. **(2017**) Melatnin vs. corn germ axes cells - modifications of antioxidant system under the chilling stress; III International Conference of Cell Biology "The versatility of cell biology research in life science" (26-27 V 2017 Cracov/Poland) Abstract 49.

*Konferencje krajowe:*

- Rakowiec M., **Kołodziejczyk I**., Szewczyk R., Posmyk M.M. **(2013)** The analsis of changes in the content of melatonin and its derivatives in corn (*Cucumis sativus* L.) seed one year after osmoconditioning; XV Ogólnopolskie Akademickie Seminarium Studentów Biotechnologii & V International Conference of Biotechnology Students (22-24 XI 2013 Wroclaw/Poland) str. 72
- **Kołodziejczyk I**., Rakowiec M., Szewczyk R., Posmyk M.M. **(2013)** The analysis of changes in the content of melatonin and its derivatives in corn (*Zea mays* L.) seed one year after hydroconditioning; XV Ogólnopolskie Akademickie Seminarium Studentów Biotechnologii & V International Conference of Biotechnology Students (22-24 XI 2013 Wrocław) str. Errata
- **Kołodziejczyk I**., Posmyk M.M. **(2014)** Rola melatoniny w organizmie roślinnym; III Studencka Konferencja Biologii Molekularnej (20-22 III 2014 Łódź) str. 61
- **Kołodziejczyk I**., Frasiński S., Posmyk M.M. **(2014)** Melatonina: jedna cząsteczka - wiele funkcji; II Łódzkie Sympozjum Doktorantów Chemii (7-8 V 2014 Łódź) str. 141.
- **Kołodziejczyk I**., Posmyk M.M. **(2014)** Hydrokondycjonowanie – efektywny zabieg polepszania wartości siewnej ziarniaków kukurydzy; Pierwsze Forum Młodych Przyrodników pt. „Rolnictwo. Żywność. Zdrowie" (14 VI 2014 Lublin) str. 80
- **Kołodziejczyk I**., Frasiński S. **(2014)** Endoreplikacja i czynniki ją regulujące; Wkraczając w Świat Nauki 2014 (11-12 IX 2014) str. 143
- Frasiński S., **Kołodziejczyk I. (2014)** Poliploidyzacja – zjawisko odpowiedzialne za korzystne cechy u roślin; Wkraczając w Świat Nauki 2014 (11-12 IX 2014) str. 128

- **Kołodziejczyk I**., (opiekun: Posmyk M.M.) **(2014)** Egzogenna melatonina jako czynnik modyfikujący metabolizm kiełkujących nasion kukurydzy (prezentacja ustna); I Ogólnopolska Konferencja Młodych Naukowców Nauk Przyrodniczych „Wkraczając w Świat Nauki 2014" (11-12 IX 2014 Wrocław) str. 51
- **Kołodziejczyk I.,** Frasiński S., Kopczewski T., Posmyk M.M. **(2014)** Roślinne białka stresowe; I Poznańskie Sympozjum Młodych Naukowców Nowe Oblicze Nauk Przyrodniczych (15 XI 2014 Poznań) str. 112
- **Kołodziejczyk I.,** Frasiński S., Kopczewski T. (opiekun: Posmyk M.M.) **(2014)** Melatonina jako biostymulator roślinny; Księga Abstraktów „Mała Wielka Nauka - Mikro nano i co dalej?" (4-5 XII 2014 Łódź) str. P-84
- **Kołodziejczyk I**., Dzitko K., Szewczyk R., Posmyk M.M. **(2015)** I. Impact of hydropriming supplemented with melatonin on corn (*Zea mays* L.) seed protein profiles during germination under optimal temperature conditions; Ogólnopolska Konferencja Naukowa "Nauka dla hodowli i nasiennictwa roślin uprawnych" (2-6 II 2015r. Zakopane) Streszczenia referatów i plakatów str. 406-407
- **Kołodziejczyk I**., Dzitko K., Szewczyk R., Posmyk M.M. **(2015)** II. Impact of hydropriming supplemented with melatonin on corn (*Zea mays* L.) seed protein profiles during germination under chilling stress conditions; Ogólnopolska Konferencja Naukowa "Nauka dla hodowli i nasiennictwa roślin uprawnych" (2-6 II 2015r. Zakopane) Streszczenia referatów i plakatów str. 408-409
- Posmyk M.M., **Kołodziejczyk I**., Bałabusta M. **(2015)** Melatonina jako biostymulator nasion – aspekt fizjologiczny i proteomiczny (prezentacja ustna); Biostymulatory w nowoczesnej uprawie roślin (25-26 II 2015 Warszawa) str. 100
- Kopczewski T., **Kołodziejczyk I**., Frasiński S. **(2015)** Rola reaktywnych form tlenu w odpowiedzi roślin na stres środowiskowy; I Toruńskie Sympozjum Doktorantów Nauk Przyrodniczych (20-22 III 2015 Toruń) str. 165
- Posmyk M.M., **Kołodziejczyk I**., Bałabusta M. **(2015)** Melatonina jako biostymulator nasion – aspekt fizjologiczny i proteomiczny; Biostymulatory w nowoczesnej uprawie roślin (25-26 II 2015 Warszawa) str. 100
- **Kołodziejczyk I.,** Dzitko K., Szewczyk R., Posmyk M.M. **(2015)** Wpływ hydroprimingu uzupełnianego melatoniną na profile białkowe nasion kukurydzy (*Zea mays* L.) podczas kiełkowania w optymalnych warunkach temperaturowych. IV Ogólnopolska Konferencja Biologii Molekularnej Łódź/Polska 26-28 III 2015 (Wydz. BiOŚ UŁ) Książka Abstraktów str. 86
- **Kołodziejczyk I**., Frasiński S. **(2015)** Endoreplikacja i poliploidyzacja w organizamach roślinnych; I Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen (20-22 IV 2015) str. 82
- **Kołodziejczyk I**., Kopczewski T. **(2015)** Pozytywne aspekty występowania endocykli w organizmach roślin użytkowych. II Seminarium Naukowe „Zielone Idee 21 Wieku" (15 X 2015r. Poznań) Materiały konferencjne (sesja posterowa)
- Szafrańska K., **Kołodziejczyk I**., Posmyk M.M. **(2015)** Czy melatonina aplikowana przedsiewnie do nasion wpływa na metabolizm związków fenolowych w liściach grochu poddanych działaniu parakwatu? II Poznanskie Sympozjum Młodych Naukowców nt. „Nowe oblicze nauk przyrodniczych" (14 XI 2015 r. Poznań) Abstrakty (poster) str. 63
- **Kołodziejczyk I**., Posmyk M.M. **(2016)** Fitobiostymulujące właściwości melatoniny – mocne wsparcie ekologicznej uprawy roślin; **Prezentacja ustna KI** na V Konferencja Biologii Molekularnej (7-9 IV 2016 Łódź) Książka Abstraktów str. 34-35
- **Kołodziejczyk I**., Posmyk M.M. **(2016)** Melatonina jako biostymulator ziarniaków kukurydzy cukrowej (*Zea mays* L.) II Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOPEN (12-14 V 2016 Łódź) Książka Abstraktów str. 158 Przewodnicząca Komitetu Organizacyjnego Konferencji

- **Kołodziejczyk I**., Posmyk M.M. **(2016)** Hydrokondycjonowanie uzupełniane melatonina - metoda przedsiewnego uszlachetniania ziaren kukurydzy cukrowej; II Ogólnopolska e-Konferencja Innowacyjnych Projektów Badawczych (2 IX 2016 Wrocław), Materiały Konferencyjne – Innowacyjne Projekty Badawcze str. 20
- **Kołodziejczyk I**., Posmyk M.M. **(2017**) Melatonina – neurohormon zwierzęcy, biostymulator roślinn; III Poznańskie Sympozjum Młodych Naukowców (5 XI 2016 Poznań)
- **Kołodziejczyk I**., Posmyk M.M. **(2017**) Melatonina, a kiełkowanie kukurydzy – modyfikacje systemu antyoksydacyjnego w stresie chłodu; XIII Ogólnopolska Konferencja Naukowa „Nauka dla hodowli i nasiennictwa roślin uprawnych" (30 I - 3 II 2017 Zakopane) Książka Abstraktów str. 236
- Szafrańska K., **Kołodziejczyk I**., Posmyk M.M. **(2017**) Melatonina utrzymuje sprawność aparatu fotosyntetycznego w stresie oksydacyjnym poprzez specyficzną regulację metabolizmu chlorofilu; XIII Ogólnopolska Konferencja Naukowa „Nauka dla hodowli i nasiennictwa roślin uprawnych" (30 I - 3 II 2017 Zakopane) Książka Abstraktów str. 251
- **Kołodziejczyk I**., Posmyk M.M. **(2017)** Modyfikacje systemy antyoksydacyjnego w zarodkach ziarniaków kukurydzy cukrowej wzbogaconych egzogenną melatoniną. III Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOPEN (11-12 V 2017 Łódź) Książka Abstraktów P.9 str. 43 Przewodnicząca Komitetu Organizacyjnego Konferencji

## 7.3. Działalność naukowa

### 7.3.1. Staże zagraniczne

Praktyki w ramach programu Erasmus +, *Plant biosystematic in the Arctic* (7 IX – 7 XI 2017, Islandzki Instytut Historii Naturalnej).

### 7.3.2. Nagrody

- Nagroda za zajęcie I miejsca w konkursie na najlepszy komunikat wygłoszony na konferencji - **Kołodziejczyk I**., Posmyk M.M. **(2015)** W jaki sposób poprawić wartość siewną nasion kukurydzy? - Melatonina jako fitobiostymulator. Prezentacja ustna KI na II Seminarium Naukowe „Zielone Idee 21 Wieku" (15 X 2015r. Poznań) Materiały konferencyjne (sesja referatowa) str. 87
- Dyplom Szczególnego Uznania za wspieranie edukacji ekologicznej w Polsce (nadany przez *Ekonatura*, kwiecień 2015)

*Załącznik 1*

Journal of Elementology ISSN 1644-2296

Kołodziejczyk I., Posmyk M.M. 2016. *Melatonin – a new plant biostimulator?* J. Elem., 21(4): 1187-1198. DOI: 10.5601/jelem.2015.20.3.1012

**REVIEW PAPER**

# MELATONIN – A NEW PLANT BIOSTIMULATOR?

**Izabela Kołodziejczyk, Małgorzata M. Posmyk**

**Department of Ecophysiology and Plant Development**
**University of Lodz**

**Abstract**

Melatonin (MEL) is a highly conserved molecule occurring in evolutionarily distant organisms such as bacteria, mono- and multicellular algae, higher plants, invertebrates and vertebrates. Although until recently, this molecule was mainly known as an animal hormone and neurotransmitter, its role in plants is currently being extensively investigated. MEL, *N*-acetyl-5--methoxytryptamine was found in various agronomically important vegetables, fruits, grains and herbs. Its concentration varies from ng to pg per gram fresh weight. MEL is present in all plant organs with its highest level found in seeds. Since the germ tissues are highly vulnerable to oxidative damage, and MEL is well known as an effective amphiphilic free radical scavenger, MEL may play an important role in plant antioxidant defence system. Especially in desiccated seed tissues, where the activity of detoxifying enzymes is limited, MEL may be essential for protecting plant germ and reproductive tissues from oxidative injuries. Recent studies provide solid evidence for MEL acting as plant growth and development regulator as well as a biostimulator, especially under unfavourable environmental conditions. Various plant species rich in MEL exhibit higher capacity for stress tolerance. MEL is also involved in stress-affected developmental transitions including flowering, fruiting and senescence. Plants are equipped with an enzymatic system for MEL biosynthesis; they are also able to synthesize a MEL precursor, tryptophan. In addition to *in vivo* synthesis, plants can also absorb exogenously provided MEL from the environment. These, and particularly the evidence that in plants MEL induces resistance against stresses, suggest that our concept of seed enrichment with exogenous MEL is justified. Our experiments proved that exogenous MEL applied into seeds by pre-sowing treatment (priming) improved their vigour and germination efficiency as well as seedling growth.

**Keywords:** MEL, phytobiostimulator, seed priming.

Izabela Kołodziejczyk, PhD student, Department of Ecophysiology and Plant Development, Faculty of Biology and Environmental Protection, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland, e-mail: izka.kolo@gmail.com

## INTRODUCTION

Melatonin is a natural compound commonly occurring in a large number of evolutionarily unrelated eukaryotic and prokaryotic taxa. It was isolated for the first time from a bovine pineal gland in 1958 and considered to be an animal neurohormone performing the critical function in circadian rhythm, seasonal reproductive cycles, and in modulating immunology in mammals (Van Tassel et al. 2001, Agozzino et al. 2003, Hernandez-Ruiz et al. 2004, Pape, Luning 2006). Subsequently, because of its numerous roles in animal organisms, considerable interest arose in MEL utilization as a therapeutic agent in many diseases such as circadian rhythm disorders, insomnia, Alzheimer's disease or even different kinds of cancer (Agozzino et al. 2003).

Since then, MEL has been searched and described in organisms belonging to all kingdoms: *Monera, Protista, Animalia, Plantae* and *Fungi (*Manchester et al. 2000). Much attention was focused on the study of *Gonyaulax polyedra (Lingulodinium polyedrum* 1987*)* belonging to dinoflagellate (Balzer, Hardeland 1991, Van Tassel et al. 2001) and then of feverfew (*Tanacetum parthenium*), St John's Wort (*Hypericum perforatum*) and other medicinal herbs (Murch, Saxena 1997). The presence of endogenous MEL in these organisms directed the attention of scientists to its role in autotrophs, especially in higher plants (Dubbels et al. 1995, Hattori et al. 1995). Since then, the presence of this indoleamine in edible plants and medicinal herbs has been extensively reported (Posmyk, Janas 2009, Paredez et al. 2009, Tan et al. 2012, Arnao 2014). Melatonin has been found *inter alia* in apple, barley, bean, cucumber, grapes, lupin, maize, potato, rice, tomato *etc.* and its concentration in plants varies from ng to pg per gram (Chen et al. 2003, Arnao, Hernández-Ruiz 2006, 2007**,** Paredes et al. 2009, Posmyk, Janas 2009, Manchester et al. 2000, Chen et al. 2009, Tan et al. 2012). It was detected and quantified in roots, shoots, leaves, flowers, fruits and seeds, but its highest level is found in reproductive organs, particularly in seeds. Since MEL was identified in a huge number of edible plants and herbs, its presence in food products and beverages originating from plants is unsurprising. Various beverages e.g. beer, coffee, red vine, and several types of tea (Tan et al. 2012) and also olive and mustard oil (Iriti et al. 2010) were studied as potential natural sources of MEL.

It was observed that MEL concentrations differed not only from species to species but also among varieties of the same species (Tan et al. 2012). It was suggested that variations in MEL contents might result not only from different extraction and detection techniques applied (i.a. radioimmunoassays, high performance liquid chromatography with electrochemical or fluorescent detection, mass spectrometry) but also from the fact that biosynthesis and metabolism of this indoleamine are affected and modified by environmental conditions (i.a. stresses), and MEL levels change during plant ontogenesis (Okazaki, Ezura 2009). It partially explains why different organs of

the same plant contain various amounts of MEL during consecutive stages of morphological and physiological development.

Generally, it was noticed that various plant species rich in MEL had greater capacity for stress tolerance (Park, Lee et al. 2013, Bajwa et al. 2014, Zhang et al. 2015) and this fact turned our attention to melatonin as a potential effective factor improving plant stress defence.

## THE ROLE OF MELATONIN IN PLANTS

It should be pointed out that evolutionary MEL is a high conserved molecule and cell protection has been its primary role. Plants possess the necessary enzymatic system for MEL biosynthesis. In contrast to animals, they are also able to synthetize the MEL precursor - tryptophan. However, in addition to *in vivo* synthesis, plants can also absorb exogenously provided MEL from the environment and accumulate its high concentrations (Tan et al. 2007*b*, Kołodziejczyk et al. 2015).

Melatonin is a structural analogue of indole-3-acetic acid (IAA) – a common auxin, hence the debate over its potential auxin-like properties is in progress. Biosynthetic pathway of both indoleamines are similar – both are derived from tryptophan. In the case of MEL, it is based on four enzymatic reactions (Figure 1). Firstly, tryptophan decarboxylase (TDC) catalyzes tryptophan transformation into tryptamine. The second step –tryptamine 5-hydroxylase (T5H) derived from cytochrome P450, hydroxylates tryptamine and converts it into serotonin. The third phase – arylalkylamine N-acetyltransferase (AANAT) catalyzes N-acetylserotonin generation in chloroplasts. Finally, N-acetylserotonin is converted into MEL using N-acetylserotonin methyltransferase (ASMT) in the cytoplasm (Kang et al. 2013, Byeon et al. 2014, Zhang et al. 2015).

Currently, the role of MEL in plant physiology is being intensively explored. Much evidence implicates MEL as a growth promoter and plant development factor (Hernández-Ruiz et al. 2004, 2005, Arnao, Hernández-Ruiz 2007, Hernández-Ruiz, Arnao 2008*a*, Chen et al. 2009, Sarrou et al. 2014, Zhang et al. 2014).

Under certain conditions MEL can simulate the auxin effects (Arnao 2014). Evidence for the involvement of MEL in root formation in *Hypericum perforatum* L. (Murch et al. 2001), and etiolated hypocotyls of *Lupinus albus* L. (Arnao, Hernandez-Ruiz 2007) was provided. A similar effect accompanied by an increase in IAA level was observed in the roots of mustard (*Brassica juncea* L. Czern.) after exogenous MEL application (Chen et al. 2009). However, it is unclear whether MEL induces auxin biosynthesis, or whether it may be metabolized and converted to IAA, or whether by structural analogy to IAA MEL exerts auxin-like effects – but these concepts need more studies.

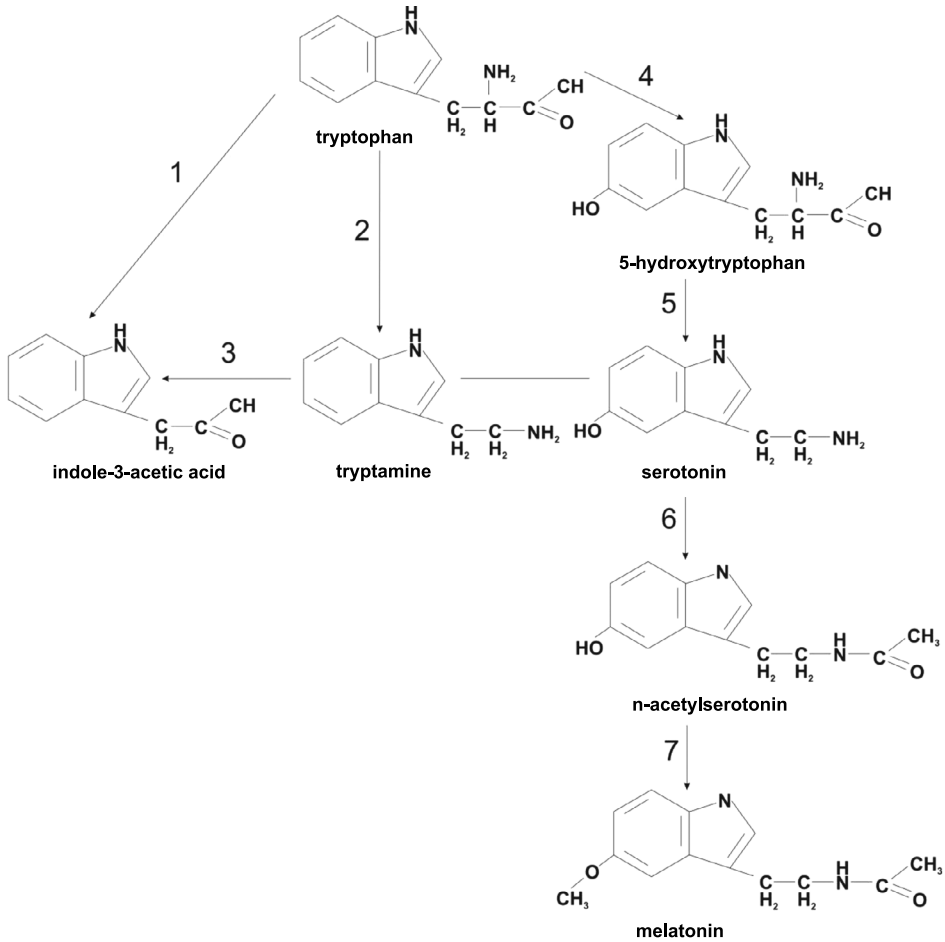



Fig. 1. Melatonin biosynthesis pathway. Enzymes involved: 1 – L-tryptophan transaminase and decarboxylase, 2 – L-tryptophan decarboxylase, 3 – tryptamine deaminase, 4 – tryptophan-5-hydroxylase, 5 – 5-hydroxytryptophan decarboxylase, 6 – serotonin N-acetyltransferase, 7 – acetyloserotonin N-methyltransferase

It was also shown that MEL in a dose-dependent manner stimulated elongation of hypocotyls (Hernández-Ruiz et al. 2004) and cotyledons of lupine – *Lupinus albus* L. (Hernández-Ruiz, Arnao 2008*c*).

Poeggeler et al. (1991) suggested participation of MEL in circadian cycle regulation in unicellular photosynthesizing dinoflagellate *Lingulodinium polyedrum*. A similar rhythm of MEL occurrence was reported in short-day plants, *e.g. Chenopodium rubrum* (Wolf et al. 2001), while daily changes in MEL content were not observed in tomato (Van Tassel et al. 2001). Research of Tan et al. (2007*a*) on the water hyacinth revealed the highest content of MEL and its metabolite N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) just before sunset, which might confirm the fact that MEL synthesis depends

on the intensity of light. This differentiates plants from animals, since in the latter light suppresses MEL production in pineal glands (Murch et al. 2000). It seems that the MEL content in plants is regulated not only by its biosynthesis rate but also by the intensity of its consumption. Thus, although the correlation of rhythmic MEL content changes with the circadian rhythm depending on the photoperiod was confirmed in some plants, this phenomenon seems to be more complex and susceptible to the influence of additional factors. In general, rhythmic changes in the MEL content in plants, like in other organisms, may be evolutionarily related to the photoperiod. Melatonin synthesized and stored in the dark is consumed in the light in order to reduce photo-oxidative damage so its level during the day falls. It seems that repeated rhythmic fluctuations associated with the synthesis and utilization of MEL could be used by organisms to biochemically measure duration of the day, and consequently to determine changes of the seasons (Kołodziejczyk et al. 2015). However, it should be taken into account that during evolution plants developed a number of other photoreceptors (phytochrome, cryptochrome, light-dependent photosynthetic apparatus), which effectively regulate the processes of photomorphogenesis.

Certainly, melatonin consumption in the light is associated with protection against photooxidizing processes, to which photosynthetic apparatus of plants is particularly exposed. In plants treated with this indoleamine photosynthesis was more efficient, concentrations of starch, sorbitol and sucrose were higher and chlorophyll degradation during the aging process was slower, while the expression of pheophorbide oxidase (*PAO*) gene (a key gene for the chlorophyll degradation process) and senescence-associated gene 12 (*SAG12)* was inhibited. Moreover, exogenous MEL inhibited the expression of sugar-sensing and senescence-associated hexokinase-1 gene *(HXK1)* (Wang et al. 2013*b*) and effectively prevented the accumulation of $H_2O_2$, which is a characteristic indicator of the cell aging process (Wang et al. 2012, 2013*a*). In *A. thaliana* MEL treatment inhibited chlorophyllase (*CLH1*) gene expression, an enzyme regulated by light and also involved in chlorophyll degradation (Weeda et al. 2014). Decreased chlorophyll degradation in barley leaves treated with MEL was also observed and its effects were more pronounced than those invoked by cytokines, widely accepted as anti-aging hormones (Arnao, Hernandez-Ruiz 2009*a*). A similar impact of exogenous MEL on chlorophyll protection against the effects of oxidative stress was observed in the leaves of *A. thaliana* treated with herbicide paraquat (Weeda et al. 2014). Studies on rice revealed that treatment with MEL significantly reduced chlorophyll degradation, suppressed transcripts of senescence-associated genes, delayed leaf senescence and enhanced salt stress tolerance (Liang et al. 2015).

Various stresses inhibit plant growth *via* different mechanisms but all stimulate reactive oxygen species (ROS) production and disturb red-ox homeostasis. It is well known that oxidative stress is a secondary effect of all biotic and some abiotic stresses. Thus, the antioxidant behaviour based on the capability of electron donation resulting in such antioxidant structure

reconformation which does not allow for secondary free radical appearance is highly desirable in plant cells. Since MEL is soluble in both water and lipids, it may be a hydrophilic and hydrophobic antioxidant. This fact together with the MEL's small size makes it particularly able to migrate easily between cell compartments in order to protect them against excessive ROS. Moreover, recent evidence indicates that the primary MEL metabolites, especially AFMK, also have high antioxidant abilities. AFMK could be formed by numerous free-radical reactions thus a single MEL molecule is reported to scavenge up to 10 ROS. It is documented that the free radical scavenging capacity of MEL extends to its secondary, tertiary and quaternary metabolites (Tan et al. 2000, 2002, 2007*c*). This process is referred to as the free radical scavenging cascade, which makes MEL even at low concentrations highly effective at protecting organisms against oxidative stress. This cascade reaction is characteristic of MEL making it more efficient than other conventional antioxidants.

As it was mentioned above, various plant species rich in MEL have shown higher capacity for stress tolerance (Park et al. 2013, Bajwa et al. 2014, Zhang et al. 2015). Exogenously applied MEL improved resistance to *Marssonina* apple blotch (Yin et al. 2013), this also implies its important role in plant innate immunity. Melatonin is also involved in stress-affected developmental transitions including flowering, fruiting and senescence (Kolar et al. 2003, Arnao, Hernández-Ruiz 2009*a*, Zhao et al. 2013, Byeon, Back 2014). An increase in the MEL content was detected in sunflower seeds during sprouting (Cho et al. 2008). Since the germ tissue is highly vulnerable to oxidative damage, MEL might be an important component of its antioxidant defense system as a free radical scavenger. Thus, MEL in seeds may be essential for protecting plant germ and reproductive tissues from oxidative injuries (Manchester et al. 2000). It have been described that plant MEL actually scavenges ROS in *Vigna radiata* leaves and protects *Gentiana macrophylla* protoplasts under UV-B exposure (Tan et al. 2012). Generally, oxidative stress elevates amount of endogenous melatonin (Boccalandro et al. 2011). Transgenic plants opulent of endogenic MEL survive negative effects induced by ROS and are able to prevent the damage (Wang et al. 2012). Moreover, it is proved that elevated concentration of melatonin ensures higher levels of GSH, vitamin C and E or carotenoids (Tan et al. 2012, Wang et al. 2012). These suggest that MEL constitutes the first line of antioxidant safeguard.

The aforementioned facts, and particularly the evidence that MEL induced resistance to stresses in plants, indicate that our concept of seed or whole plant enrichment with exogenous MEL is justified.

# MELATONIN – A NOVEL PLANT BIOSTIMULATOR

Plant biostimulators – phytostimulators are various kinds of non-toxic substances of natural origin that improve and stimulate plant life processes differently than fertilizers or phytohormones. Their influence on plants is not the consequence of direct metabolic regulation of particular processes but their action could be multidirectional and they influence metabolism more generally. The crucial point is that biostimulators, in contrast to bioregulators, improve plant metabolic processes without changing their natural pathway.

Recent plant neurobiology trends in science based on auxin signaling investigations (Brenner et al. 2006) focus our attention on MEL, which demonstrates parallelism with the plant auxin – indole-3-acetic acid (IAA). Resumption of research on classic plant models used in plant physiology is still necessary to clarify the role and mechanism of action of MEL: (i) as an independent plant growth regulator, (ii) as a factor mediating the activity of other substances influencing plant growth or (iii) as a substance involved in growth regulation but whose activity generally is ascribed to other compounds. However, recent knowledge may qualify MEL as a biostimulator. Its advantages are as follows: (i) it is of natural origin but it can be easily synthesized in laboratories, (ii) it is non-toxic, (iii) it is not expensive, (iv) it dissolves in different solvents: water, alcohols but also lipids – which facilitates the use of various application methods (v) it could be actively uptaken by plants from environment, (vi) it is a small molecule easily penetrating cell compartments, (vii) it has strong antioxidant properties (viii) it improves plant tolerance to stresses.

The quality of seed material is a primary and basic condition determining good harvest. Thus, finding effective methods to improve sowing material by applying biostymulators into seeds is a crucial problem. Generally, it was observed that physiological concentrations of MEL in seeds were very high, for example in white and black mustard seeds they were 129 and 189 ng $g^{-1}$, respectively (Hattori et al. 1995), much higher than the known physiological blood concentrations of many vertebrates

The known techniques of seed priming: hydro- and osmo-conditioning were tested by Posmyk and colleagues (Posmyk, Janas 2007, Posmyk et al. 2008, 2009, 2009*a*, Janas et al. 2009). Different pre-sowing seed treatments effectively counteract diseases and pests as well as improve seed viability and seedling vigour *per se* (Taylor et al. 1998). All of them are based on controlled seed hydration. These techniques can be combined with other supporting factors such as aeration, light-irradiation, temperature-stratification. Seed priming can also be combined with an application of growth regulators and other bioactive substances (McDonald 1999). Our previous experiments (Posmyk, Janas 2007, Posmyk et al. 2008, 2009, 2009*a*, Janas et al. 2009) proved that exogenous MEL applied into seeds by pre-sowing treatment improved their vigour and germination as well as seedling growth.

Indeed, red cabbage seed hydropriming with MEL proved to be a good tool for vigour improvement (Posmyk et al. 2008). Positive effect of this treatment was visible especially under copper stress conditions. Similarly, experiments with cucumber seeds osmoprimed with MEL (Posmyk et al. 2009*a*, 2009) and with corn seeds hydroprimed with MEL (Janas et al. 2009) proved its positive effects on seeds.

Beneficial effects of priming were not visible in cucumber and corn seed germination tests performed under optimal temperature conditions. Except that primed seeds germinated more uniformly (smaller ±SEM), both parameters: the germination rate and the final germination percentage, were comparable with control seeds (Posmyk et al. 2009*a*, Janas et al. 2009). However, subsequent experiments showed that seedlings grown from seeds conditioned with melatonin tolerated better stresses of suboptimal temperature (10°C) and heavy metal contamination (2.5 mM $Cu^{2+}$), and also regenerated much better after the relief of stress. It was manifested by better growth (greater weight of seedlings) and higher chlorophyll content and phenolic synthesis in the seedlings grown from seeds hydroprimed with melatonin (Janas et al. 2009).

When in seeds laboratory tests were subjected to stress, differences between the untreated and conditioned variants were apparent. Cucumber seeds osmoprimed with MEL started to germinate at 10°C, which was impossible for the control, untreated ones (Posmyk et al. 2009, 2009*a*). We also obtained interesting results when corn seeds were germinated at 10°C (Janas et al. 2009 ). The control non-primed seeds germinated after 5 days of imbibition and achieved ~97% germinability after 12 days of experiments, whereas those treated with MEL reduced the lag phase up to 2 days and all seeds were germinated on day 10 of experiment. Hydropriming without MEL also reduced the lag phase to 3 days of imbibition but germinability of the seeds decreased to ~90% (Janas et al. 2009). The seed pre-sowing treatments accelerated germination rates of corn seeds at suboptimal temperature.

Field tests equivocally proved that MEL applied in a specific way to seeds was a perfect biostimulator enhancing plant growth and development as well as increasing yield. Field experiments were performed with the following seeds: *Cucumis sativus* L., *Zea mays* L, *Vigna radiata* L. (Janas, Posmyk 2013). All seeds were primed and re-dried under laboratory conditions, up to one month before being sown in fields. During vegetation, the plants were not supplemented by fertilizers or pesticides – experiments were performed as organic farming. The field experiments showed that plants from the MEL -treated seeds of corn, mung bean and cucumber were greater, better developed, their vegetation was prolonged (they were longer green) and they had higher crop yield than the control ones (Posmyk, Janas 2009, Janas, Posmyk 2013). At harvesting, 50 μM MEL osmoprimed cucumber plants had more fruits than those osmoprimed with MEL 500 μM, osmoprimed without MEL or non-treated plants. We observed that some fruits of MEL-treated plants

were larger than those of osmoprimed without MEL and non-osmoprimed ones (Janas, Posmyk 2013). 50 and 500 μM MEL-treated corn plants had more and larger cobs than those hydroprimed without MEL and the non-primed plants. Similar results were observed on mung bean, whose seeds were hydroprimed with MEL at 20, 50 and 500 μM concentrations. Numbers of pods were greater in the plants grown from the seed hydroprimed with 50 μM MEL than in hydroprimed without MEL and non-primed ones (Posmyk, Janas 2009, Janas, Posmyk 2013). It is surprising that a single MEL application into the seeds increased yield of plants growing naturally in a field. It was assessed that the production of corn, cucumber and mung bean primed with MEL was about 10-25% greater in comparison to those primed without MEL and it depended on plant species (Janas, Posmyk 2013).

Since melatonin is safe for animals and humans as well as inexpensive, a conditioning technique using this indoleamine as a plant biostimulator may be a reliable, feasible and cost-effective tool for positive seed quality modification and may be economically beneficial for organic farming (Posmyk, Janas 2009, Janas, Posmyk 2013).

## AKNOWLEDMENTS

The topic was orally presented during 2nd International Conference: *Biostimulators in modern plant cultivation* (25 - 26.02.2015 r. Warsaw/Poland). We are grateful to the Conference Organizers for supporting and funding this publication.

## REFERENCES

Agozzino P., Avellone G., Bongiorno D., Ceraulo L., Filizzola F., Natoli M.C., Livrea M.A., Tesoriere L. 2003. *Melatonin: structural characterization of its non-enzymatic mono-oxygenate metabolite.* J. Pineal Res., 35: 269-275.

Arnao M.B., Hernandez-Ruiz J. 2006. *The physiological function of melatonin in plants.* Plant Sign. Behav., 1(3): 89-95.

Arnao M.B., Hernandez-Ruiz J. 2007. *The melatonin promotes adventitious and lateral root regeneration in etiolated hypocotyls of Lupinus albus* L. J. Pineal Res., 42: 147-152.

Arnao M.B., Hernandez-Ruiz J. 2009a. *Protective effect of melatonin against chlorophyll degeneration during the senescence of barley leaves.* J. Pineal Res., 46: 58-63.

Arnao M.B., Hernández-Ruiz J. 2009b. *Assessment of different sample processing procedures applied to the determination of melatonin in plants.* Phytochem. Anal., 20: 14-18.

Arnao M.B., Hernández-Ruiz J. 2014. *Melatonin: plant growth regulator and/or biostimulator during stress?* Trends Plant Sci., 19: 789-97.

Arnao M.B. 2014. *Phytomelatonin: discovery, content, and role in plants.* Adv. Bot. Article ID 815769. DOI: 10.1155/2014/815769

Bajwa V.S., Shukla M.R., Sherif S.M. 2014. *Role of melatonin in alleviating cold stress in Arabidopsis thaliana.* J. Pineal Res., 56: 238-245.

Balzer I., Hardeland R. 1991. *Photoperiodism and effects of indoleamines in a unicellular alga, Gonyaulax polyedra.* Science, 253: 795-797.

Boccalandro H.E., Gonzales C.V., Wunderlin D.A., Fernanda M. 2011. *Melatonin levels*

*determined by LC-ESI-MS/MS, deeply fluctuate during the day in Vitis vinifera cv Malbec. Evidences of its antioxidant role in fruits*. J. Pineal Res., 51: 226-232.

Brenner E.D., Stahlberg R., Mancuso S., Vivanco J., Baluška F., Van Volkenburgh E. 2006. *Plant neurobiology: an integrated view of plant signaling*. Trends Plant Sci., 11: 413-419.

Byeon Y., Back K.W. 2014. *An increase in melatonin in transgenic rice causes pleiotropic phenotypes, including enhanced seedling growth, delayed flowering, and low grain yield*. J. Pineal Res., 56: 380-414.

Byeon Y., Lee H.Y., Lee K., Park S., Back K.W. 2014. *Cellular localization and kinetics of the rice melatonin biosynthetic enzymes SNAT and ASMT*. J. Pineal Res., 56: 107-114.

Chen G.F., Huo Y.S., Tan D-X., Liang Z., Zhang W., Zhang Y. 2003. *Melatonin in Chinese medicinal herbs*. Life Sci., 73: 19-26.

Chen Q., Qi W., Reiter R.J., Wei W., Wang B. 2009. *Exogenously applied melatonin stimulates root growth and raises endogenous indoleacetic acid in roots of etiolated seedlings of Bassica juncea*. J. Plant Physiol., 166: 324-328.

Cho M.H., No H.K., Prinyawiwatkul W. 2008. *Chitosan treatments affect growth and selected quality of sunflower sprouts*. J. Food Sci., 73: 70-77.

Dubbels R., Reiter R.J., Klenke E., Goebel A., Schnakenberg E., Ehlers C., Schiwara H.W., Schloot W. 1995. *Role melatonin in edible plants identified by radioimmunoassay and high performance liquid chromathography-mass spectrometry*. J. Pineal Res., 18: 28-31.

Hattori A., Migitaka H., Iigo M., Itoh M., Yamamoto K., Ohtani-Kaneko R., Hara M., Suzuki T., Reiter R.J. 1995. *Identification of melatonin in plants and its effect on plasma melatonin levels and binding to melatonin receptors in vertebrates*. Biochem. Mol. Biol. Int., 35: 627-634.

Hernandez-Ruiz J., Cano A., Arnao M.B. 2004. *Melatonin: a growth-stimulating compound present in lupin tissues*. Planta, 220: 140-144.

Hernández-Ruiz J., Cano A., Arnao M.B. 2005. *Melatonin acts as a growth-stimulating compound in some monocot species*. J. Pineal Res., 39: 137-142.

Hernández-Ruiz J., Arnao M.B. 2008*a*. *Melatonin stimulates the expansion of etiolated lupin cotyledons*. Plant Growth Regul., 55: 29-34

Hernández-Ruiz J., Arnao M.B. 2008*b*. *Distribution of melatonin in different zones of lupin and barley plants at different ages in the presence and absence of light*. J. Agr. Food Chem., 56: 10567-10573.

Hernández-Ruiz J., Arnao M.B. 2008*c*. *Melatonin stimulates the expansion of etiolated lupin cotyledons*. Plant Growth Regul., 55: 29-34.

Iriti M., Varoni E.M., Vitalini S. 2010. *Melatonin in traditional Mediterranean diets*. J. Pineal Res., 49: 101-105.

Janas K.M., Ciupińska E., Posmyk M.M. 2009. *Melatonin applied by hydropriming, as phytobiostimulator improving corn (Zea mays* L.*) seedlings growth at abiotic stresses conditions*. In: *Progress in environmental science and technology*. Li S., Wang Y., Cao F., Huang P., Zhang Y. (Eds.). Vol II A. Science Press USA Inc., 383-388.

Janas K.M., Posmyk M.M. 2013. *Melatonin, an underestimated natural substance with great potential for agricultural application*. Acta Physiol. Plant, 35: 3285-3292.

Kang K., Lee K., Park S., Beon Y., Back K. 2013. *Molecular cloning of rice serotonin N-acetyltransferase, the penultimate gene in plant melatonin biosynthesis*. J. Pineal Res., 55: 7-13.

Kolar J., Johnson C.H., Machackova I. 2003. *Exogenously applied melatonin (N-acetyl--5-methoxytryptamine) affects flowering of the short-day plant Chenopodium rubrum*. Physiol. Plant., 118: 605-612.

Kołodziejczyk I., Bałabusta M., Szewczyk R., Posmyk M.M. 2015. *The levels of matonin and its metabolites in conditioned corn (Zea mays* L.*) and cucumber (Cucumis sativus* L.*) seeds during storage*. Acta Phys. Plant., 37: 105.

Liang C., Zheng G., Li W., Wang Y., Wu H., Qian Y., Zhu X.G., Tan D.X., Chen S.Y., Chu C. 2015. *Melatonin delays leaf senescence and enhances salt stress tolerance in rice.* J. Pineal Res., 59(1): 91-101. DOI: 10.1111/jpi.12243

Manchester L.C., Tan D.X., Reiter R.J., Park W., Monis K., Qi W.B. 2000. *High levels of melatonin in the seeds of edible plants – possible function in germ tissue protection.* Life Sci., 67: 3023-3029.

McDonald M.B. 1999. *Seed priming.* In: *Seed technology and its biological basis.* Black M., Bewley J.D. (Eds.) Sheffield Academic Press, Sheffield, UK, 287-325.

Murch S.J., Saxena P.K. 1997. *Melatonin in feverfew and other medical plants.* The Lancet, 350(29): 1598-1999.

Murch S.J., Krishna R.S., Saxena P.K. 2000. *Tryptophan is a precursor for melatonin and serotonin biosynthesis in in vitro regenerated St. John's Wort (Hypericum perforatum* L. *cv. Anthos).* Plant Cell Rep., 19: 698-704.

Murch S.J., Campbell S.S.B., Saxena P.K. 2001. *The role of serotonin and melatonin in plant morphogenesis: regulation of auxin-induced root organogenesis in in vitro cultured explants of St. John's Wort (Hypericum perforatum* L.*).* In Vitro Cell Dev. Biol., 37:786-793.

Okazaki M., Ezura H. 2009. *Profiling of melatonin in the model tomato (Solanum lycopersicum* L.*) cultivar Micro-Tom.* J. Pineal Res.*,* 46: 338-343.

Pape C., Luning K. 2006. *Quantification of melatonin in phototrophic organisms.* J. Pineal Res., 41: 157-165.

Paredes S.D., Korkmaz A., Manchester L.C., Tan D-X., Reiter R.J. 2009. *Phytomelatonin: a review.* J. Exp. Bot., 60(1): 57-69.

Park S., Lee D.E., Jang H., Byeon Y., Kim Y.S., Back K. 2013. *Melatonin rich transgenic rice plants exhibit resistance to herbicide-induced oxidative stress.* J. Pineal Res., 54: 258-263.

Poeggeler B., Balzer I., Hardeland R., Lerchi A. 1991. *Pineal hormone melatonin oscillates also in the Dinoflagellate Gonyaulax polyedra.* Naturwissenschaften, 78: 268-269.

Posmyk M.M., Janas K.M. 2007. *Effect of seed hydropriming in presence of exogenous proline on chilling injury limitation in Vigna radiata* L. *seedlings.* Acta Physiol. Plant., 29: 509-517.

Posmyk M.M., Kuran H., Marciniak K., Janas K.M. 2008. *Pre-sowing seed treatment with melatonin protects red cabbage seedlings against toxic copper ion concentration.* J. Pineal Res., 45: 24-31.

Posmyk M.M., Bałabusta M., Wieczorek M., Śliwińska E., Janas K.M. 2009. *Melatonin applied to cucumber (Cucucmis sativus* L.*) seeds improves germination during chilling stress.* J. Pineal Res., 46: 214-223.

Posmyk M.M., Janas K.M. 2009. *Melatonin in plants.* Acta Physiol. Plant., 31: 1-11.

Posmyk M.M., Bałabusta M., Janas K.M. 2009*a*. *Melatonin applied by osmopriming, as phytobiostimulator improving cucumber (Cucumis sativus* L.*) seedlings growth at abiotic stresses conditions.* In: *Progress in Environmental Science and Technology.* Li S., Wang Y., Cao F., Huang P., Zhang Y. (Eds.). Vol II A. Science Press USA Inc., 362-369.

Sarrou E., Therios I., Dimassi-Theriou K. 2014. *Melatonin and other factors that promote rooting and sprouting of shoot cuttings in Punica granatum cv.* Wonderful. Turk J. Bot., 38: 293-301.

Tan D.X., Manchester L.C., Reiter R.J., QI W., Karbownik M., Calvo J.R. 2000. *Significance of melatonin in antioxidative defence system: reactions and products.* Biol. Signals Recept., 9: 137-159.

Tan D.X., Reiter R.J., Manchester L.C., Mei-Ting Y., El-Sawi M., Sainz R.M., Mayo J.C., Kohen R., Allegra M., Hardeland R. 2002. *Chemical and physical properties and potential mechanisms: melatoninas a broad spectrum antioxidant and free radical scavenger.* Curr. Topics Med. Chem., 2: 181-197.

Tan D.X., Manchester L.C., Di Mascio P., Martinez G.R., Prado F.M., Reiter R.J. 2007*a*. *Novel rhythms of N1-acetyl-N2-formyl-5-methoxykynuramine and its precursor melatonin in water hyacinth: importance for phytoremediation.* FASEB J., 21: 1724-1729.

Tan D.X., Manchester L.C., Helton P., Reiter R.J. 2007*b*. *Phytoremediative capacity of plants enriched with melatonin*. Plant Signal Behav., 2: 514-516.

Tan D.X., Manchester L.C., Terron M.P., Flores L.J., Reiter R.J. 2007*c*. *One molecule, many derivatives: A never-ending interaction of melatonin with reactive oxygen and nitrogen species?* J. Pineal Res., 42: 28-42.

Tan D.X., Hardeland R., Manchester L.C., Korkmaz A., Ma S., Rosales-Corral S., Reiter R.J. 2012. *Functional roles of melatonin in plants, and perspectives in nutritional and agricultural science*. J. Exp. Bot., 63(2): 577-597.

Taylor A.G., Allen P.S., Bennett M.A., Bradford K.J., Burris J.S., Misra M.K. 1998. *Seed enhancement*. Seed Sci. Res., 8: 245-256.

Van Tassel D.L., Roberts N., Lewy A., O'Neill S.D. 2001. *Melatonin in plant organs*. J. Pineal Res., 31: 8-15.

Wang P., Yin L., Liang D., Li C., Ma F., Yue Z. 2012. *Delayed senescence of apple leaves by exogenous melatonin: towards regulating the ascorbate-glutathione cycle*. J. Pineal Res., 53: 11-20.

Wang P., Sun X., Li C.H., Wei Z., Liang D., Ma F. 2013*a*. *Long-term exogenous application of melatonin delays drought-induced leaf senescence in apple*. J. Pineal Res., 54: 292-302.

Wang P., Sun X., Chang C., Feng F., Liang D., Cheng L., Ma F. 2013*b*. *Delay in leaf senescence of Malus hupehensis by long-term melatonin application is associated with its regulation of metabolic status and protein degradation*. J. Pineal Res., 55: 424-434.

Weeda S., Zhang N., Zhao X., Ndip G., Guo Y., Buck G.A., Fu C., Ren S. 2014. *Arabidopsis transcriptome analysis reveals key roles of melatonin in plant defense systems*. PLOS ONE, 9(3): E93462. DOI: 10.1371/journal.pone.0093462

Wolf K., Kolář J., Witters E., Van Dongen W., Van Onckelen H., Macháčková I. 2001. *Daily profile of melatonin levels in Chenopodium rubrum L. depends on photoperiod*. J. Plant Physiol., 158: 1491-1493.

Yin L., Wang P., Li M., Ke X., Li C., Liang D., Wu S., Ma X., Li C., Zou Y., Ma F. 2013. *Exogenous melatonin improves Malus resistance to Marssonina apple blotch*. J. Pineal Res., 54: 426-434.

Zhang N., Zhang H.J., Zhao B., Sun Q.Q., Cao Y.Y., Li R., Wu X.X., Weeda S., Li L., Ren S., Reiter R.J., Guo Y.D. 2014. *The RNA-seq approach to discriminate gene expression profiles in response to melatonin on cucumber lateral root formation*. J. Pineal Res., 56: 39-50.

Zhang N., Sun Q., Zhang H. 2015. *Roles of melatonin in abiotic stress resistance in plants*. J. Exp. Bot., 66: 647-656.

Zhao Y., Tan D-X., Lei Q., Chen H., Wang L., Li Q.T., Gao Y., Kong J. 2013. *Melatonin and its potential biological functions in the fruits of sweet cherry*. J. Pineal Res., 55: 79-88.

Oświadczenia współautorów publikacji **Kołodziejczyk I., Posmyk M.M. (2016)** Melatonin - a new plant biostimulator? *Journal of Elementology* 21(4): 1187-1197 DOI: 10.5601/jelem. 2015.20.3.1012 – wchodzącej w skład rozprawy doktorskiej:

| Imię i nazwisko | Szacunkowy udział [%] | Opis działań | Podpis |
|---|---|---|---|
| mgr Izabela Kołodziejczyk | 90 | Przygotowanie przeglądu literaturowego i koncepcji publikacji. Zredagowanie manuskryptu. | Kołodziejczyk Izabela |
| prof. dr hab. Małgorzata M. Posmyk | 10 | Pomoc przy redakcji i korekta manuskryptu. | |
| Dane biblio-metryczne z dn. 19.05.2021 r. wg. *Web.Sci.*: **IF 0.641**$^{2016}$**, 0.781**$^{V}$**; 14** cytowań. (**40**$^{A}$ pkt-y MEiN) | | | |

***Załącznik 2***

Acta Physiol Plant (2015) 37:105
DOI 10.1007/s11738-015-1850-7

ORIGINAL PAPER

# The levels of melatonin and its metabolites in conditioned corn (*Zea mays* L.) and cucumber (*Cucumis sativus* L.) seeds during storage

**Izabela Kołodziejczyk[1] · Marta Bałabusta[1] · Rafał Szewczyk[2] · Małgorzata M. Posmyk[1]**

Received: 12 January 2015 / Revised: 21 April 2015 / Accepted: 22 April 2015


**Abstract** The efficiency of pre-sowing conditioning/priming methods used to apply melatonin into seeds was verified: osmopriming in the case of dicot *Cucumis sativus* and hydropriming of monocot *Zea mays* seeds. Both priming techniques were selected experimentally as optimal for the studied plant species. Four different seed variants were compared: control non-treated ones, and seeds conditioned with water or with 50 or 500 μM melatonin water solutions. The HPLC–MS quantitative and qualitative analyses were used to determine the content of melatonin and of its potential metabolites in the seeds during 1 year following the conditioning. The control seeds and those conditioned with water contained small amount of endogenous melatonin in both species. However, the level of this indoleamine increased markedly in cucumber and corn seeds primed with exogenous melatonin and it was always correlated with the concentration of melatonin applied. It was noted that melatonin was metabolized during seed storage by its gradual oxidation, thus it protects dry seeds against oxidative stress, prevents potential injuries and significantly increases seeds quality. Interestingly, in the control and water-primed seeds, seasonal fluctuations of endogenous melatonin concentration were noted and significant increase in this indoleamine in the winter month was observed. This suggests that in seeds endogenous melatonin could play a crucial role in seasonal rhythms independently of environmental conditions.

**Keywords** Melatonin · Seeds · Melatonin metabolites · Seed conditioning · Plant rhythms

Communicated by J. van Staden.

✉ Małgorzata M. Posmyk
posmyk@biol.uni.lodz.pl

[1] Department of Ecophysiology and Plant Development, Faculty of Biology and Environmental Protection, University of Lodz, 12/16 Banacha Str., 90-237 Lodz, Poland

[2] Department of Industrial Microbiology and Biotechnology, Faculty of Biology and Environmental Protection, University of Lodz, 12/16 Banacha Str., 90-237 Lodz, Poland

## Introduction

Melatonin is an indoleamine (*N*-acetyl-5-methoxytryptamine) that occurs in evolutionary distant organisms. It reflects the conservative nature of this molecule. Till now, it has been found in bacteria, algae, vascular plants, invertebrates and vertebrates (Murch and Saxena 2002; Hardeland and Poeggeler 2003).

Lerner et al. (1958) identified the chemical structure of melatonin in 1958. Since then, the functions, biosynthesis and degradation of melatonin in animals, especially vertebrates, have been widely studied, well documented and accepted as being valid. As concerns the plant kingdom first melatonin was detected in the photosynthesizing alga *Gonyaulax polyedra* (Balzer and Hardeland 1991) and then in vascular plants (Dubbels et al. 1995; Hattori et al. 1995). Up till now, the occurrence of this indoleamine in many edible plants and herbs has been widely reported (Posmyk and Janas 2009; Paredes et al. 2009; Tan et al. 2012; Arnao 2014). Melatonin was found in fresh fruits/plants and food products, e.g., in tomatoes, cherries, olives and oils, grapes and red vine, walnuts, sunflower, mustard oil, etc, and its content varies from nanograms to picograms per gram of these products (Chen et al. 2003; Arnao and Hernández-Ruiz 2006, 2009b; Hernández-Ruiz and Arnao 2008b; Paredes et al. 2009; Posmyk and Janas 2009). Moreover, melatonin was detected and quantified in different plant

Published online: 07 May 2015

organs: roots, shoots, leaves, flowers, fruits and seeds, but its highest concentrations were noted in reproductive organs, particularly in seeds.

Currently, melatonin functions in plant physiology are being intensively explored. Some evidence implicates its role as a growth stimulator and plant development factor (Hernández-Ruiz et al. 2004, 2005; Arnao and Hernández-Ruiz 2007; Hernández-Ruiz and Arnao 2008a; Chen et al. 2009; Sarrou et al. 2014; Zhang et al. 2014). It seems that this indoleamine plays a significant role in plant stress defense. It was observed that melatonin-rich plant species showed higher capacity for tolerance of unfavorable conditions (Park et al. 2013; Bajwa et al. 2014; Zhang et al. 2015). Moreover, exogenously applied melatonin improved resistance to *Marssonina* apple blotch caused by *Diplocarpon mali*—dangerous pathogen that generates losses in the cultivation of apples (Yin et al. 2013). This implies the role of the described indoleamine also in combat against biotic stresses. The importance of melatonin in stress effects/injuries limitation during different developmental stages including flowering, fruiting, and senescence was discussed (Kolar et al. 2003; Arnao and Hernández-Ruiz 2009a; Zhao et al. 2013; Byeon and Back 2014). An increase in melatonin level in sunflower seeds during sprouting was also observed (Cho et al. 2008). Since the germ tissue is highly susceptible to oxidative injuries, melatonin in seeds might act as an important antioxidant and effective free radical scavenger. Thus, melatonin may be essential for protecting plant germ and reproductive tissues from oxidative damages (Manchester et al. 2000).

Plants, in contrast to animals, are able to synthesize the melatonin precursor—tryptophan, so they provide complete biosynthetic pathway leading to endogenous melatonin accumulation. In addition to unlimited in vivo synthesis, plants can also absorb exogenous melatonin from the environment (Tan et al. 2007). This and particularly the evidence that melatonin induces resistance to stresses in plants indicate that our concept of seed enrichment with exogenous melatonin is justified. Our previous experiments (Posmyk et al. 2008, 2009a, b; Janas et al. 2009) proved that exogenous melatonin applied into seeds by pre-sowing treatment improved their vigor and germination as well as seedling growth. Since melatonin is nontoxic and biodegradable, thus safe for the environment, as well as being inexpensive, a priming methods supplemented with this indoleamine may be a reliable, feasible and cost-effective tool for seed quality improvement thus beneficial for organic farming (Janas and Posmyk 2013).

Generally, seed priming comprises different conditioning techniques (e.g., hydro-, osmo-, matri-conditioning) based on controlled seed hydration leading to limited seed imbibition. This pre-sowing treatment can be supplemented with an application of growth stimulators, protective components and other bioactive substances. In the presented work, the efficacy of two techniques used for melatonin application into seeds was tested. The following priming methods were verified: osmopriming (O) in the case of dicot *Cucumis sativus* and hydropriming (H) in the case of monocot *Zea mays*. The conditioning methods were selected experimentally as optimal for seeds of the studied species. In the case of both plants, four variants of seeds were compared: non-treated control (C), and seeds conditioned with water or conditioned with 50 and 500 μM melatonin water solutions. Melatonin content in the seeds and the appearance of its potential metabolites were monitored during 1 year following the conditioning.

## Materials and methods

### Plant material

The seeds of cucumber (*Cucumis sativus* L. var. Odys) and corn (*Zea mays* L. var. Ambrozja) were provided by 'Torseed' (Torun, Poland). All seeds (different variants) were stored in dry conditions, at room temperature, in the dark, in tightly closed containers.

### Hydropriming

Corn seeds were hydroprimed by soaking them at 25 °C in darkness for 3 h, using distilled water or melatonin (50 or 500 μM) water solutions. Seed water content was increased from an initial value of 8.8 % (±0.2) to 36.8 % (±2.3). The quantity of water needed to increase seed humidity was determined experimentally. Corn seed variants: control (C)—non-treated, hydroprimed (H), hydroprimed with melatonin 50 and 500 μM (HMel50 and HMel500, respectively). Seeds were re-dried during 3 days at room temperature after hydropriming.

### Osmopriming

Cucumber seeds were osmoprimed as described by Posmyk et al. (2001). Seeds were placed on a layer of cotton wool wet with polyethylene glycol-8000 (PEG-8000) solution at −1.5 MPa for 4 days. The PEG osmotic potential was calculated according to Michael and Kaufman (1973); PEG was dissolved in distilled water or melatonin solutions to final concentrations 50 and 500 μM. Treatments were carried out in darkness at 25 °C. Seed water content was increased from an initial 8.10 % (±1.3) to final 27.8 % (±1.4). Cucumber seed variants: control (C)—non-treated, osmoprimed (O), osmoprimed with melatonin, 50 and 500 μM (OMel50 and OMel500, respectively). Seeds were

re-dried during 3 days at room temperature after osmopriming.

## Melatonin and its derivatives determination in seeds

### *Extraction procedure*

The seeds (0.5 g) were homogenized in ice bath, with 6 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA and 5 μM butylated hydroxytoluene (BHT) as an antioxidant. The homogenate was incubated for 15 min at room temperature in darkness with minimal shaking, to ensure complete extraction of indoles. It was subsequently centrifuged at 20,000*g* for 10 min at 5 °C. The supernatant was filtered through two layers of Miracloth and purified in two steps by solvent partitioning using ethyl acetate (first at pH 8.0 and second at pH 3). Each 3 ml of supernatant was shaken horizontally with 10 ml of ethyl acetate (15 min/darkness/room temperature). The first organic phase was collected. The remaining aqueous phase was adjusted to pH 3.0 with 1N $H_3PO_4$, supplemented with the next 10 ml of ethyl acetate followed by horizontal shaking (30 min/darkness/room temperature). The second organic phase was added to the first one and both were evaporated together under vacuum. Dry residue was redissolved in 100 μl of acetonitrile (ACN) and then top up to 1 ml with deionized water and filtered through Supelco ISO-Disc filters (PTEF-4-2.4 mm, Å ~ 0.2 μm) before HPLC–MS analysis. The extraction efficiency was estimated as ~85 %.

### *HPLC–MS analyses*

The concentrations of melatonin and its metabolites were determined in plant tissue extracts using high-performance liquid chromatography with mass spectral detection

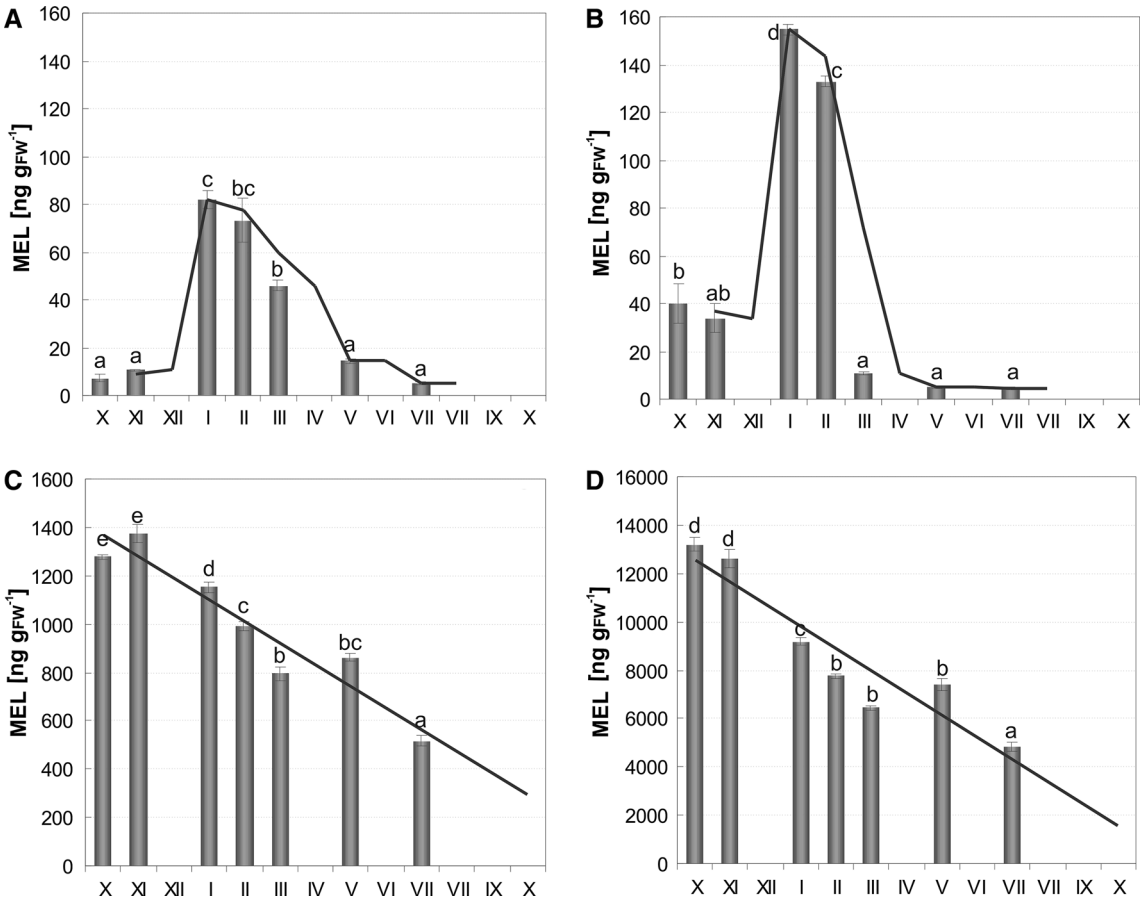



**Fig. 1** Changes in melatonin level of dry cucumber (*Cucumis sativus* L.) seeds, beginning from osmopriming and indoleamine application (X—October) and during the following months of seed storage: control non-treated seeds (**a**), seeds osmoprimed with water (**b**), seeds osmoprimed with melatonin at the concentrations of 50 (**c**) or 500 (**d**) μM. The results are expressed as mean values of six measurements ±SEM. One-way ANOVA and Bonferroni's post hoc test were performed. The *small letters* next to the values show statistical significance $p < 0.001$. ANOVA results: **a** months of measurements (X, XI, I, II, III, V, VII) $F(6.11) = 58.71$; **b** months of measurements (X, XI, I, II, III, V, VII) $F(6.12) = 257.02$; **c** months of measurements (X, XI, I, II, III, V, VII) $F(6.32) = 135.62$; **d** months of measurements (X, XI, I, II, III, V, VII) $F(6.34) = 184.45$

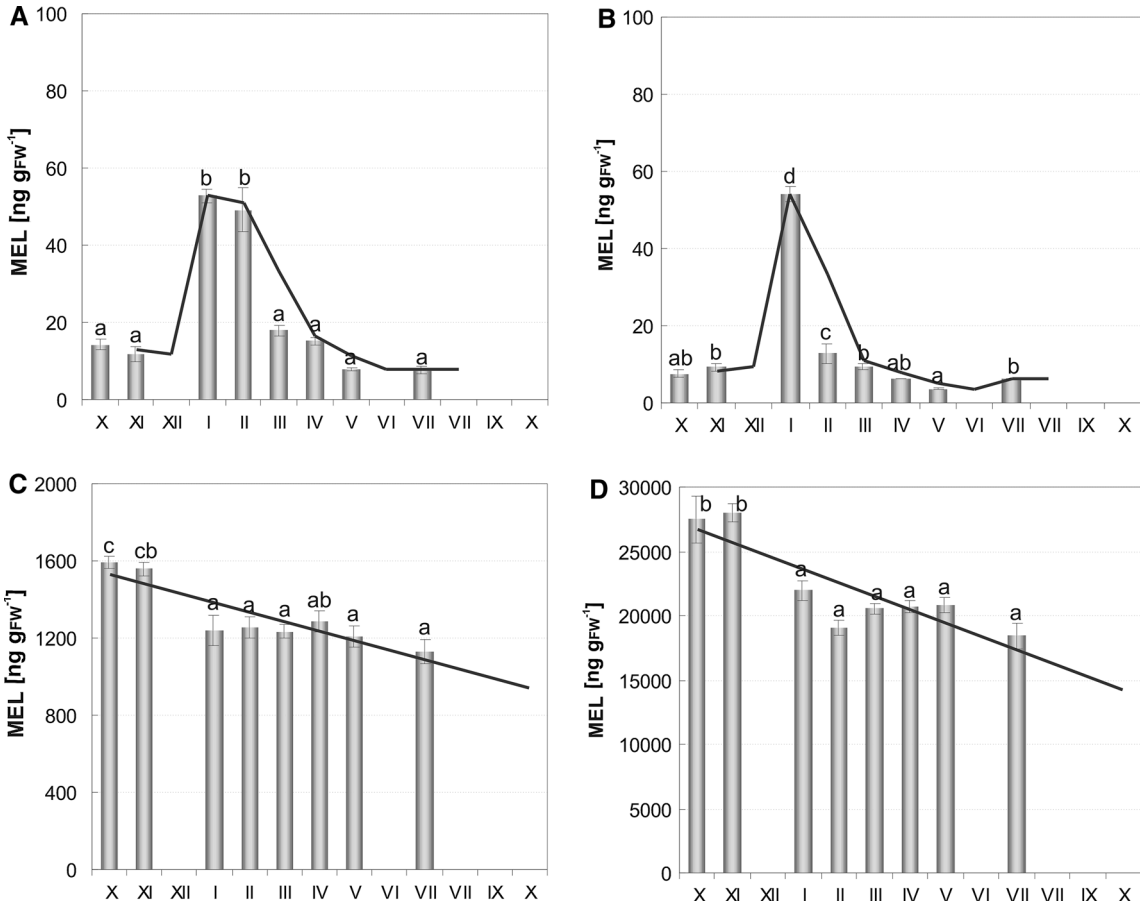



**Fig. 2** Changes in melatonin level of dry corn (*Zea mays* L.) seeds beginning from hydropriming and indoleamine application (X—October) and during the following months of seed storage: control non-treated seeds (**a**), seeds hydroprimed with water (**b**), seeds hydroprimed with melatonin at the concentrations of 50 (**c**) or 500 (**d**) μM. The results are expressed as mean values of six measurements ±SEM. One-way ANOVA and Bonferroni's post hoc test were performed. The *small letters* next to the values show statistical significance $p < 0.001$. ANOVA results: **a** months of measurements (X, XI, I, II, III, IV, V, VII) $F(7) = 50.94$; **b** months of measurements (X, XI, I, II, III, IV, V, VII) $F(7) = 200.67$; **c** months of measurements (X, XI, I, II, III, IV, V, VII) $F(7) = 8.29$; **d** months of measurements (X, XI, I, II, III, IV, V, VII) $F(7) = 13.75$

(HPLC–MS). Analyses were performed on Agilent 1200 LC System coupled with AB Sciex 3200 QTRAP mass detector equipped with TurboSpray Ion Source (ESI). The column used was Agilent SB-C18, 1.8 μm, 4.6 × 50 mm and a mobile phase was a mixture of: A—$H_2O$ + 2 mM ammonium formate + 0.2 % formic acid; B—ACN + 2 mM ammonium formate + 0.2 % formic acid.

*Quantitative analysis*

10 μl of the tested samples was injected on a column maintained in isocratic conditions A:B—20:80, temperature 40 °C and 600 μl $min^{-1}$ flow. The retention time of melatonin was 7.69 min. MS/MS detection was done in MRM positive ionization mode. Optimized MRM pairs for melatonin were: 233.2–174.2 *m/z* (CE = 17)—quantifier ion, 233.2–130.1 (CE = 57)—qualifier ion. The other parameters of the detector were: CUR: 35.00; TEM: 600.00; GS1: 50.00; GS2: 60.00; ihe: ON; IS: 5500.00; CAD: Medium; DP: 36.00; EP: 5.50; CEP: 14.00; CXP: 3.00. Standard equation used for the quantitative analysis showed linearity in the range from 0 to 100 ng $ml^{-1}$ of melatonin ($r = 0.9992$).

*Qualitative analysis*

10 μl of the tested samples was injected on a column with mobile phase flow 500 μl $min^{-1}$ and temperature set to 40 °C. The following gradient was applied: 0—1 min 90:10 (A:B); 1—15 min gradient 90:10 (A:B) to 10:90 (A:B) in 15 min and maintained to 20 min; 20.10 reverse to start conditions 90:10 (A:B) and maintained till the end of the method in 23.00 min. MS/MS detection was made in IDA (information-dependant acquisition) mode composed of mixed scan modes and IDA criteria for dynamic *m/z* filtering. The method was constructed as follows: Prec 1

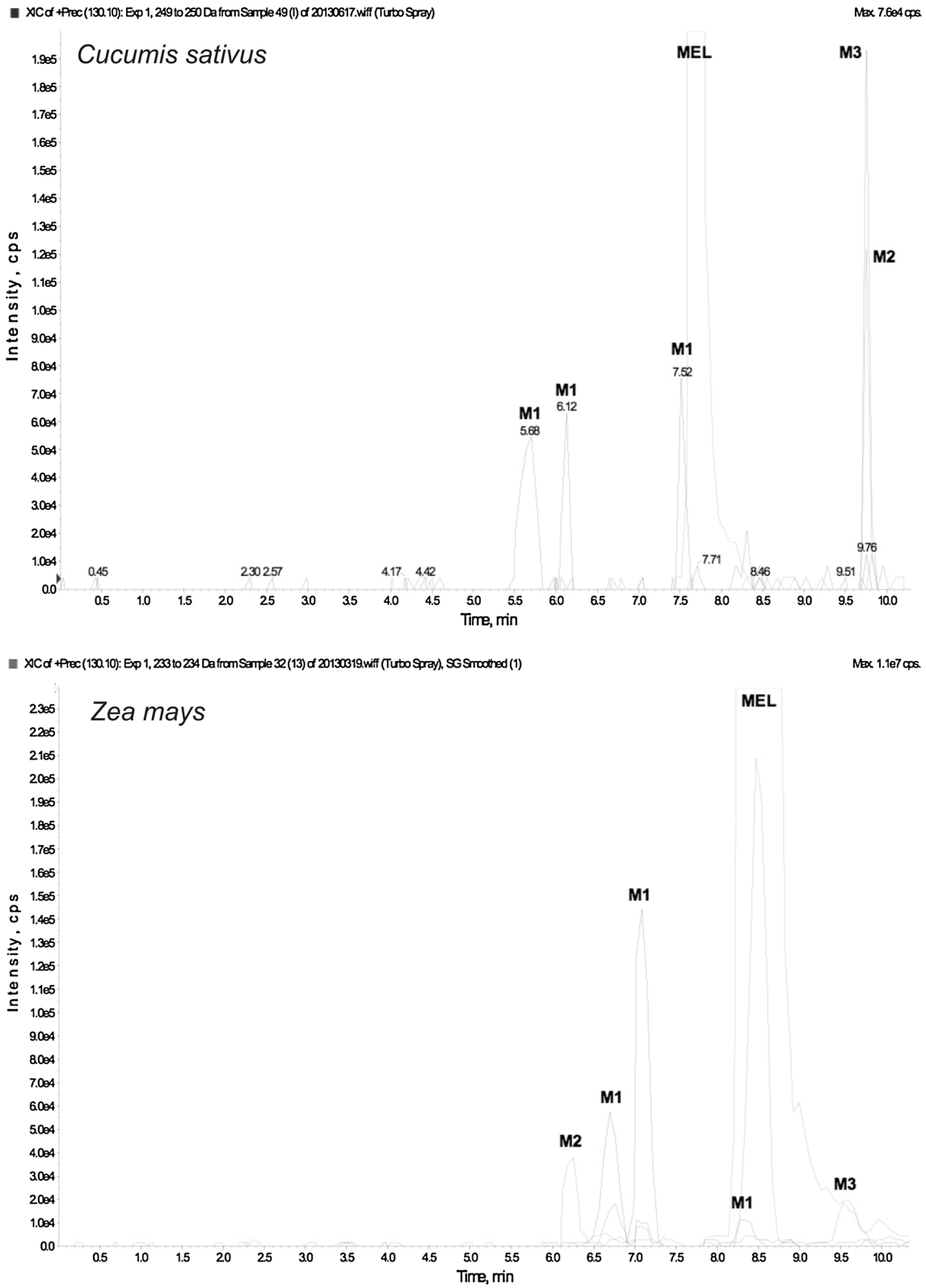



**Fig. 3** Chromatographic analysis of melatonin (MEL) and its metabolites (M1-3) detected in *Cucumis sativus* L. and *Zea mays* L. seeds preconditioned (osmoprimed and hydroprimed, respectively) with melatonin. Analyses were performed after 10 months of dry seed storage

(Precursor ion scan), Prec 2, ER (enhanced resolution scan), IDA criteria, EPI (enhanced product ion scan). Precursors were 130.1 *m/z* (DP = 36, CE = 50) working in range 140–350 *m/z* and 174.2 *m/z* (DP = 36, CE = 17) working in range 188–400 *m/z*. Both precursors were assigned as markers for potential melatonin metabolites. ER scan was working in 250 Da/s scan rate (DP = 36) and was used for isotopic distribution studies of molecular ion species. EPI scan was working in range 50–400 *m/z* (DP = 36, CE = 50, CES = 15) and was used for $MS^2$ mass spectra collection. The other MS parameters were the same as in quantitation method. The most important IDA criteria used for selective *m/z* filtering were as follows: choose 1–2 most intense peaks from range 130–400 *m/z* which charge state is +1 and exceeds 10,000 counts intensity, exclude former target ions for 30 s after three occurrences.

### Statistic analysis

The results are expressed as mean values of six measurements ±SEM. The data were analyzed using STATISTICA v.10.0_MR1_PL [StatSoft] software. One-way analysis of variance (ANOVA) and then the post hoc Bonferroni's multiple range test was carried out to find the significant differences at $p < 0.001$ in each experiment and variant.

## Results and discussion

The present research focused on changes in melatonin content in dry cucumber and corn seeds. Physiological concentrations of this indoleamine in the control seeds, at the beginning of the experiment, were low and persisted in the ranges 7–11 ng $g_{FW}^{-1}$ in cucumber (Fig. 1a) and 12–14 ng $g_{FW}^{-1}$ in corn (Fig. 2a) seeds. Both species of seeds untreated with exogenous melatonin (i.e., the control and those osmo- or hydroprimed with water) despite constant storage conditions (tightly sealed plastic bags, darkness, room temperature) showed seasonal changes in this indoleamine concentration with significant increases in the winter months (Figs. 1a, b, 2a, b). These seasonal changes—noted in the first time in seeds—may suggest the existence of an endogenous circannual rhythm regulated/set by melatonin—independently of environmental conditions. By definition, an endogenous rhythm is one in which the cycle persists although external conditions remain constant.

The possible role of melatonin as a regulator of light/dark cycles in plants also has been studied. Some oscillations in endogenous melatonin levels have been described in the green macroalgae *Ulva* sp. (Tal et al. 2011) and also in higher plants: *Chenopodium rubrum* L. (Kolar et al. 1995, 1997; Wolf et al. 2001), *Eichhornia crassipes* (hyacinth) (Tan et al. 2007), *Vitis vinifera* (Boccalandro et al. 2011), and *Prunus* sp. (Zhao et al. 2013), pointing to its possible role as a regulator of light/dark-mediated rhythms. The changes mentioned above were associated with the external factor—photoperiod. Our results suggest the existence of an independent 'chemical memory' in plants that is not susceptible to extrinsic factors. Seasonal fluctuations in the amount of melatonin in seeds indicate existence of endogenous biological clock that functions as a biochemical calendar (probably developed evolutionarily, e.g., in cold and temperate climate changes) precisely regulated by this melatonin.

Both conditioning techniques, osmopriming and hydropriming, when melatonin was present proved to be highly effective tools allowing this indoleamine to accumulate in the seeds of cucumber and corn, respectively. In both species, levels of this compound increased proportional to the concentration of melatonin applied and became much higher than its physiological levels in both cucumber (Fig. 1a, c, d) and corn (Fig. 2a, c, d) seeds. However, in both variants of seeds treated with exogenous melatonin, a continuous decline of this indoleamine was observed throughout the experiment: from October 2013 to July 2014 (Figs. 1c, d, 2c, d).

Derivatives of the applied melatonin began to emerge along with a reduction of melatonin content in the seeds. Chromatographic analysis of melatonin and its metabolites in *Cucumis sativus* L. revealed three derivatives (Fig. 3 *Cucumis sativus*: M1, M2, M3). In all cases, the melatonin molecule was oxidized by addition of a hydroxyl (–OH) group to the second carbon (β) in the side chain of the hetero-ring, e.g., like in M1 (Fig. 4a–c). Additionally, elimination of the acetamide ($-NH-CO-CH_3$) group and oxidation to the aldehyde in M2 was observed (Fig. 4b), or the third ring was undergoing cyclization as seen in M3 (Fig. 4c).

Chromatographic analysis of melatonin and its metabolites in *Zea mays* L. also revealed three derivatives (Fig. 3 *Zea mays*: M1, M2, M3). In the formation of M1 and M2, melatonin was oxidized by addition of a hydroxyl (–OH) group to the second carbon (β) in the side chain of hetero-ring (Fig. 5a, b); additionally, in M2 this side chain was reduced to two carbons and the acetamide (–NH–CO–$CH_3$) group was removed (Fig. 5b). In the third derivative, M3, only cyclization of the third ring was observed (Fig. 5c).

The IDA method for substrate metabolite screening was developed according to the workflow described elsewhere and successfully applied for biodegradation studies of alachlor (Słaba et al. 2013). On the basis of the EPI and $MS^3$ (data not shown) scans of melatonin, the fragmentation pathway leading to full interpretation of melatonin mass spectra was done (Fig. 6). The obtained data were

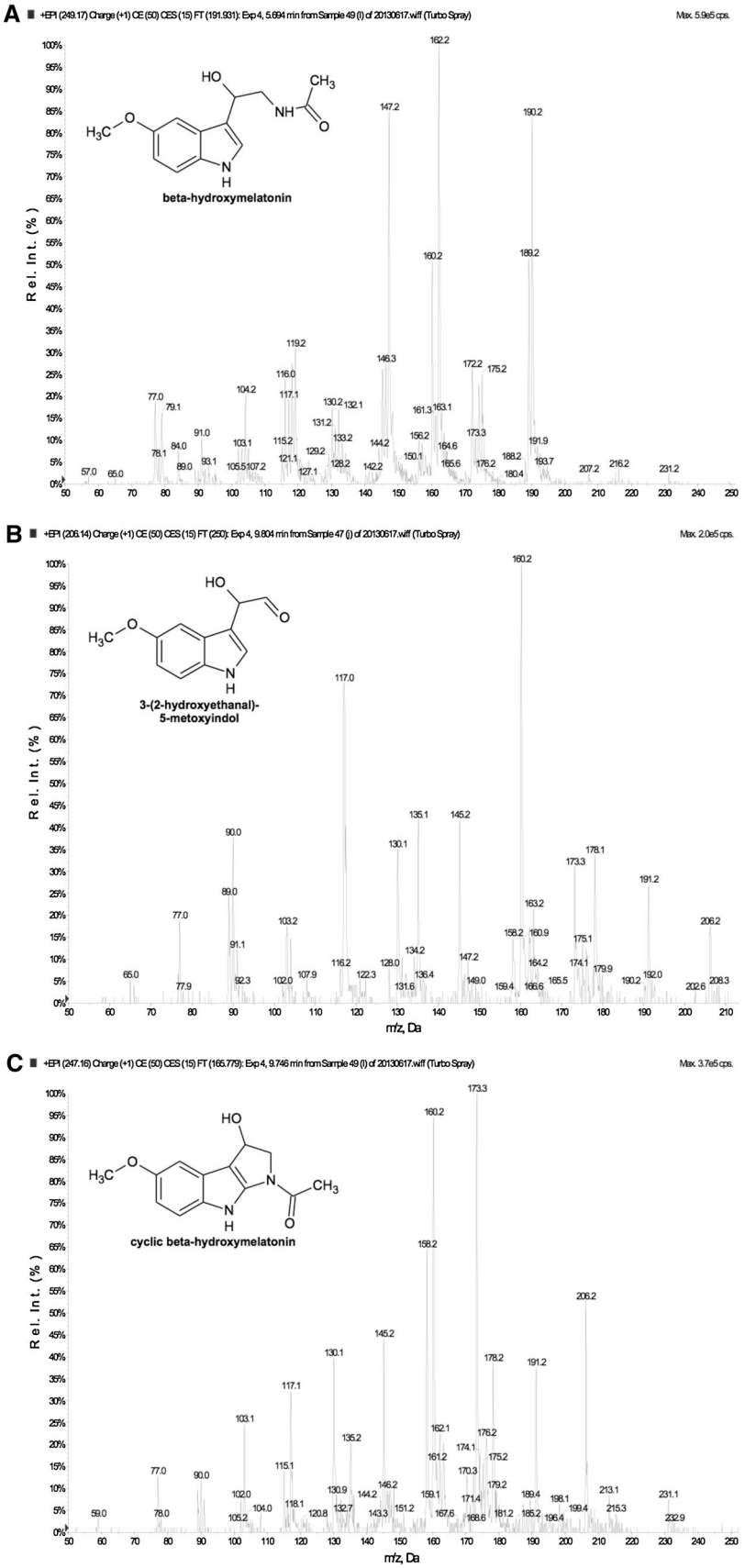



**Fig. 4** Mass spectra of particular metabolites detected in *Cucumis sativus* L. seeds after 10 months of storage: M1 = beta-hydroxymelatonin (**a**), M2 = 3-(2-hydroxyethanal)-5-metoxyindol (**b**), M3 = cyclic beta-hydroxymelatonin (**c**)

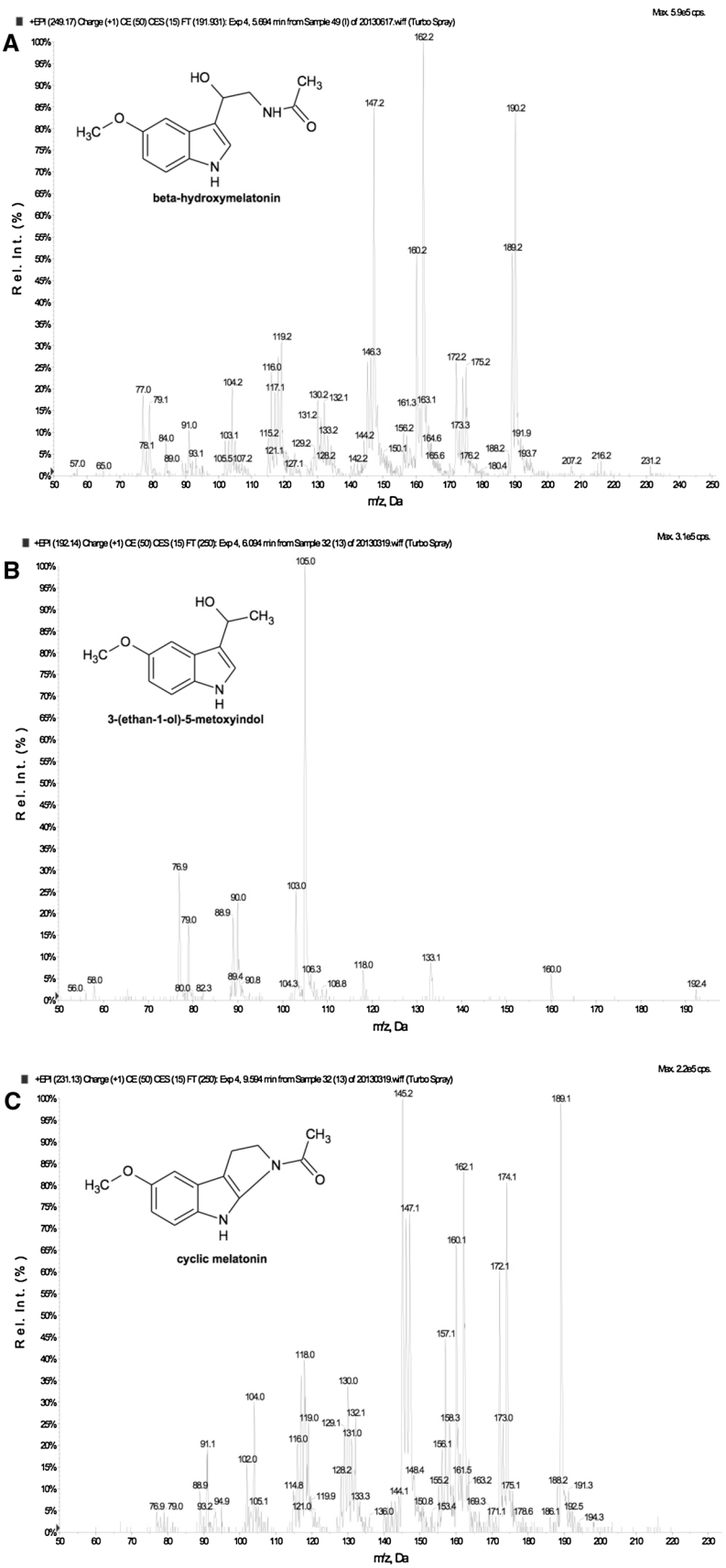



**Fig. 5** Mass spectra of particular metabolites detected in *Zea mays* L. seeds after 10 months of storage: M1 = beta-hydroxymelatonin (**a**), M2 = 3-(ethan-1-ol)-5-metoxyindol (**b**), M3 = cyclic melatonin (**c**)

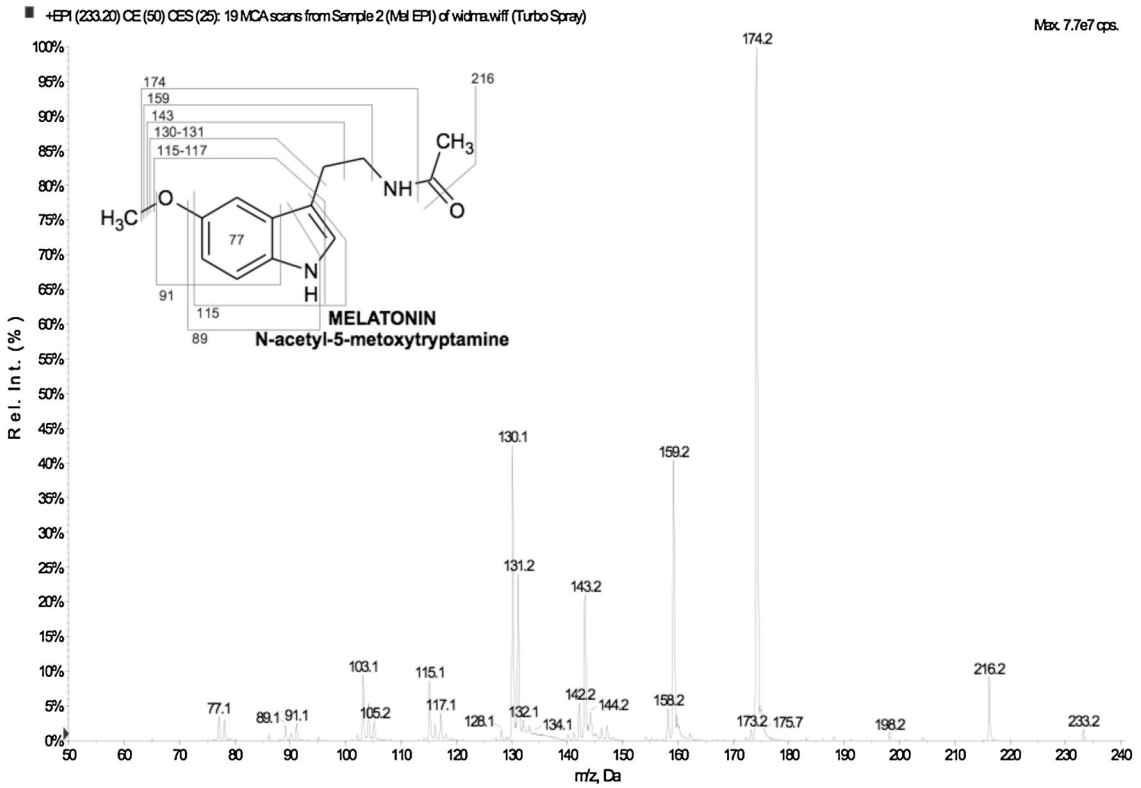



**Fig. 6** Melatonin mass spectrum (pattern)

used for the construction of semi-targeted IDA method scanning the samples for potential melatonin metabolites, on the basis of precursor ion scans 130.1 *m/z* ($C_9H_7N^+$, 3-methyl-1H-indole substructure) and 174 *m/z* ($C_{11}H_{11}NO^+$, 3-ethyl-5-methoxy-1H-indole substructure). Based on a comparative mass spectra analysis of the samples, 6 metabolites were found. All of them underwent characteristic fragmentation in the EPI scan confirming the presence of 3-ethyl-5-methoxy-1H-indole substructure (normal or cyclic form). Other changes in the structure were characterized by manual mass spectra analysis supported by ACD Labs MS Fragmenter and AB Sciex PeakView 2.1 software tools.

In both plant species, the melatonin derivative labeled M1 is the same compound, β-hydroxymelatonin. Also, the pair labeled as M2 is very similar in structure. In both, hetero-ring's side chain reduction to two carbons was observed and these derivatives differed only by the degree of oxidation. In cucumber, it was 3-(2-hydroxyethanal)-5-metoxyindol and in corn 3-(ethan-1-ol)-5-metoxyindol. The third derivative, M3 in both, cucumber and corn seeds, was: cyclic β-hydroxymelatonin and cyclic melatonin, respectively.

The present data prove that the selected seed conditioning techniques to be effective tools allowing to the introduction of exogenous melatonin into both types of seeds: monocot corn grains and dicot cucumber seeds. During seed storage, the quantity of melatonin decreased considerably compared to the initial amount determined immediately after priming. The magnitude and the rate of its decline were characteristic of the species. All the metabolites were oxidized melatonin derivatives. This suggests that during seed storage melatonin is oxidized, even in relatively dry seed tissues (probably without active enzymes) and thus it protects dry seeds against oxidative stress, prevents potential injuries and significantly increases seeds quality. These positive effects were also visible as better vigor and stress tolerance of the seedlings grown from the seeds conditioned with melatonin (Posmyk et al. 2009b; Janas et al. 2009).

Important new data obtained from this work consist in evidence suggesting the existence of endogenous circannual rhythm regulated/set by the endogenous melatonin level in cucumber and corn seeds. This raises the question whether the application of exogenous melatonin in doses significantly exceeding its physiological level may impair the aforementioned rhythm? The answer will be possible only when sites of melatonin biosynthesis in plant cells and its localisation in organs and tissues of seeds are clearly established.

It is postulated that mitochondria and plastids are the primary sites of melatonin biosynthesis (Tan et al. 2013),

but during evolution some of the genes of mitochondrial and plastid genome could have been successfully transferred to the nucleus (Adams and Palmer 2003), and melatonin biosynthesis ability could have been transferred to other cell compartments such as cytosol. Studies on transgenic rice plants that examined location of typical enzymes of melatonin biosynthesis pathway in plants (De Luca and Cutler 1987; Stevens et al. 1993; Kang et al. 2007; Fujiwara et al. 2010; Zhao et al. 2013; Byeon et al. 2014) provided interesting results, indirectly confirming the hypothesis concerning correlation of melatonin biosynthesis sites with the endosymbiotic theory (symbiogenesis). It is suggested that melatonin biosynthesis in plants occurs sequentially in three cellular compartments: cytoplasm, endoplasmic reticulum (ER) and chloroplasts. Unfortunately, there is no direct evidence for visualizing its location in seed or whole plant organs and tissues. This would probably be the key information allowing to answer the question whether the application of exogenous melatonin into seeds disrupts the rhythms potentially generated by endogenous phytomelatonin. If endogenous melatonin is synthesized and stored in a specific part of an embryo and there are also the receptors regulating the annual rhythms mentioned above (which is very likely because an embryo is the most important and the only growing organ of a seed), introduction/application of exogenous melatonin, even at a high dose, stored just under the seed coat and/or in seed storage tissues should have no impact on these rhythms. However, to explain this phenomenon, precise immunohistochemical localization of melatonin in the organs and tissues of the seed is required. This will not be easy due to the co-existence of a number of structural analogs of melatonin in plants (auxins). Lack of appropriate commercial monoclonal antimelatonin antibodies for use in studying plant tissues is another serious obstacle.

**Author contribution statement** Izabela Kołodziejczyk was involved in all experiments concerning corn seeds, data acquisition and analysis. Marta Bałabusta was involved in all experiments concerning cucumber seeds, data acquisition and analysis. Rafał Szewczyk was involved in melatonin and its metabolites HPLC–MS assays. Małgorzata M. Posmyk sketched work concept and was involved in data analysis and interpretation, drafting of the manuscript.

**Acknowledgments** Research work financed in the years 2011/2014 by the National Science Centre of Poland as NCN N N310 111940 project.

## References

Adams KL, Palmer JD (2003) Evolution of mitochondrial gene content: gene loss and transfer to the nucleus. Mol Phylogenet Evol 29:380–395

Arnao MB (2014) Phytomelatonin: discovery, content, and role in plants. Adv Bot. doi:10.1155/2014/815769

Arnao MB, Hernández-Ruiz J (2006) The physiological function of melatonin in plants. Plant Signal Behav 1:89–95

Arnao MB, Hernández-Ruiz J (2007) Melatonin promotes adventitious and lateral root regeneration in etiolated hypocotyls of *Lupinus albus* L. J Pineal Res 42:147–152

Arnao MB, Hernández-Ruiz J (2009a) Protective effect of melatonin against chlorophyll degradation during the senescence of barley leaves. J Pineal Res 46:58–63

Arnao MB, Hernández-Ruiz J (2009b) Assessment of different sample processing procedures applied to the determination of melatonin in plants. Phytochem Anal 20:14–18

Bajwa VS, Shukla MR, Sherif SM, Murch SJ, Saxena PK (2014) Role of melatonin in alleviating cold stress in *Arabidopsis thaliana*. J Pineal Res 56:238–245

Balzer I, Hardeland R (1991) Photoperiodism and effects of indoleamines in a unicellular alga, *Gonyaulax polyedra*. Science 253:795–797

Boccalandro HE, Gonzalez CV, Wunderlin DA, Silva MF (2011) Melatonin levels, determined by LC–ESI–MS/MS, fluctuate during the day/night cycle in *Vitis vinifera* cv Malbec: evidence of its antioxidant role in fruits. J Pineal Res 51:226–232

Byeon Y, Back KW (2014) An increase in melatonin in transgenic rice causes pleiotropic phenotypes, including enhanced seedling growth, delayed flowering, and low grain yield. J Pineal Res 56:380–414

Byeon Y, Lee HY, Lee K, Park S, Back K (2014) Cellular localization and kinetics of the rice melatonin biosynthetic enzymes SNAT and ASMT. J Pineal Res 56:107–114

Chen GF, Huo YS, Tan D-X, Liang Z, Zhang W, Zhang Y (2003) Melatonin in Chinese medicinal herbs. Life Sci 73:19–26

Chen Q, Qi WB, Reiter RJ, Wei W, Wang BM (2009) Exogenously applied melatonin stimulates root growth and raises endogenous indoleacetic acid in roots of etiolated seedlings of *Brassica juncea*. J Plant Physiol 166:324–328

Cho MH, No HK, Prinyawiwatkul W (2008) Chitosan treatments affect growth and selected quality of sunflower sprouts. J Food Sci 73:70–77

De Luca V, Cutler AJ (1987) Subcellular localization of enzymes involved in indole alkaloid biosynthesis in *Catharanthus roseus*. Plant Physiol 85:1099–1102

Dubbels R, Reiter RJ, Klenke E, Goebel A, Schnakenberg E, Ehlers C, Schiwara HW, Schloot W (1995) Melatonin in edible plants identified by radioimmunoassay and by high performance liquid chromatography-mass spectrometry. J Pineal Res 18:28–31

Fujiwara T, Maisonneuve S, Isshiki M, Mizutani M, Chen L, Wong HL, Kawasaki T, Shimamoto K (2010) Sekiguchi lesion gene encodes a cytochrome P450 monooxygenase that catalyzes conversion of tryptamine to serotonin in rice. J Biol Chem 285:11308–11313

Hardeland R, Poeggeler B (2003) Non-vertebrate melatonin. J Pineal Res 34:233–241

Hattori A, Migitaka H, Iigo M, Itoh M, Yamamoto K, Ohtani-Kaneko R, Hara M, Suzuki T, Reiter RJ (1995) Identification of melatonin in plants and its effects on plasma melatonin levels and binding to melatonin receptors in vertebrates. Biochem Mol Biol Int 35(3):627–634

Hernández-Ruiz J, Arnao MB (2008a) Melatonin stimulates the expansion of etiolated lupin cotyledons. Plant Growth Regul 55:29–34

Hernández-Ruiz J, Arnao MB (2008b) Distribution of melatonin in different zones of lupin and barley plants at different ages in the presence and absence of light. J Agr Food Chem 56:10567–10573

Hernández-Ruiz J, Cano A, Arnao MB (2004) Melatonin: a growth stimulating compound present in lupine tissues. Planta 220:140–144

Hernández-Ruiz J, Cano A, Arnao MB (2005) Melatonin acts as a growth-stimulating compound in some monocot species. J Pineal Res 39:137–142

Janas KM, Posmyk MM (2013) Melatonin, an underestimated natural substance with great potential for agricultural application. Acta Physiol Plant 35:3285–3292

Janas KM, Ciupińska E, Posmyk MM (2009) Melatonin applied by hydropriming, as phytobiostimulator improving corn (*Zea mays* L.) seedlings growth at abiotic stresses conditions. In: Li S, Wang Y, Cao F, Huang P, Zhang Y (eds) Progress in environmental science and technology, vol IIA. Science Press, USA, pp 383–388

Kang S, Kang K, Lee K, Back K (2007) Characterization of rice tryptophan decarboxylases and their direct involvement in serotonin biosynthesis in transgenic rice. Planta 227:263–272

Kolar J, Machackova I, Illnerova H, Prinsen E, Van Dongen W, Van Onckelen HA (1995) Melatonin in higher plant determined by radioimmunoassay and liquid chromatography mass spectrometry. Biol Rhythm Res 26:406–409

Kolar J, Machackova I, Eder J, Prinsen E, Van Dongen W, Van Onckelen H, Illnerová H (1997) Melatonin: occurrence and daily rhythm in *Chenopodium rubrum*. Phytochem 44(8):1407–1413

Kolar J, Johnson C, Machackova I (2003) Exogenously applied melatonin (*N*-acetyl-5-methoxytryptamine) affects flowering of the short day plant *Chenopodium rubrum*. Physiol Plant 118:605–612

Lerner AB, Case DJ, Takahashi Y (1958) Isolation of melatonin a pineal factor that lightens melanocytes. J Am Chem Soc 80:2587

Manchester LC, Tan D-X, Reiter RJ, Park W, Monis K, Qi W (2000) High levels of melatonin in the seeds of edible plants—possible function in germ tissue protection. Life Sci 67:3023–3029

Michael BE, Kaufman MR (1973) The osmotic potential of polyethylene glycol 6000. Plant Physiol 51:914–916

Murch SJ, Saxena PK (2002) Melatonin: a potential regulator of plant growth and development? In Vitro Cell Dev Biol Plant 38:531–536

Paredes SD, Korkmaz A, Manchester LC, Tan D-X, Reiter RJ (2009) Phytomelatonin: a review. J Exp Bot 60(1):57–69

Park S, Lee DE, Jang H, Byeon Y, Kim YS, Back K (2013) Melatonin rich transgenic rice plants exhibit resistance to herbicide-induced oxidative stress. J Pineal Res 54:258–263

Posmyk MM, Janas KM (2009) Melatonin in plants. Acta Physiol Plant 31:1–11

Posmyk MM, Corbineau F, Vinel D, Bailly C, Côme D (2001) Osmoconditioning reduces physiological and biochemical damage induced by chilling soybean seeds. Physiol Plant 111:473–482

Posmyk MM, Kuran H, Marciniak K, Janas KM (2008) Pre-sowing seed treatment with melatonin protects red cabbage seedlings against toxic copper ion concentrations. J Pineal Res 45:24–31

Posmyk MM, Bałabusta M, Wieczorek M, Sliwinska E, Janas KM (2009a) Melatonin applied to cucumber (*Cucumis sativus* L.) seeds improves germination during chilling stress. J Pineal Res 46:214–223

Posmyk MM, Bałabusta M, Janas KM (2009b) Melatonin applied by osmopriming, as phytobiostimulator improving cucumber (*Cucumis sativus* L.) seedlings growth at abiotic stresses conditions. In: Li S, Wang Y, Cao F, Huang P, Zhang Y (eds) Progress in environmental science and technology, vol IIA. Science Press, USA, pp 362–369

Sarrou E, Therios I, Dimassi-Theriou K (2014) Melatonin and other factors that promote rooting and sprouting of shoot cuttings in *Punica granatum* cv. Wonderful Turk J Bot 38:293–301

Słaba M, Szewczyk R, Piątek MA, Długoński J (2013) Alachlor oxidation by the filamentous fungus *Paecilomyces marquandii*. J Haz Mat 261:443–450

Stevens LH, Blom TJM, Verpoorte R (1993) Subcellular localization of tryptophan decarboxylase, strictosidine synthase and strictosidine glucosidase in suspension cultured cells of *Catharanthus roseus* and *Tabernaemontana divaricata*. Plant Cell Rep 12:573–576

Tal O, Haim A, Harel O, Gerchman Y (2011) Melatonin as an antioxidant and its semi-lunar rhythm in green macroalga *Ulva* sp. J Exp Bot 62(6):1903–1910

Tan D-X, Manchester LC, Di Mascio P, Martinez GR, Prado FM, Reiter RJ (2007) Novel rhythms of *N*-1-acetyl-*N*-2-formyl-5-methoxykynuramine and its precursor melatonin in water hyacinth: importance for phytoremediation. FASEB J 21:1724–1729

Tan D-X, Hardeland R, Manchester LC, Korkmaz A, Ma S, Rosales-Corral S, Reiter RJ (2012) Functional roles of melatonin in plants, and perspectives in nutritional and agricultural science. J Exp Bot 63(2):577–597

Tan D-X, Manchester LC, Liu X, Rosales-Corral SA, Acuna-Castroviejo D, Reiter RJ (2013) Mitochondria and chloroplasts as the original sites of melatonin synthesis: a hypothesis related to melatonin's primary function and evolution in eukaryotes. J Pineal Res 54:127–138

Wolf K, Kolar J, Witters E, Van Dongen W, Van Onckelen H, Machackova I (2001) Daily profile of melatonin levels in *Chenopodium rubrum* L. depends on photoperiod. J Plant Physiol 158(11):1491–1493

Yin L, Wang P, Li M, Ke X, Li C, Liang D, Wu S, Ma X, Li C, Zou Y, Ma F (2013) Exogenous melatonin improves *Malus* resistance to Marssonina apple blotch. J Pineal Res 54:426–434

Zhang N, Zhang HJ, Zhao B, Sun QQ, Cao YY, Li R, Wu XX, Weeda S, Li L, Ren S, Reiter RJ, Guo YD (2014) The RNA-seq approach to discriminate gene expression profiles in response to melatonin on cucumber lateral root formation. J Pineal Res 56:39–50

Zhang N, Sun Q, Zhang H, Cao Y, Weeda S, Ren S, Guo YD (2015) Roles of melatonin in abiotic stress resistance in plants. J Exp Bot 66(3):647–656

Zhao Y, Tan D-X, Lei Q, Chen H, Wang L, Li QT, Gao Y, Kong J (2013) Melatonin and its potential biological functions in the fruits of sweet cherry. J Pineal Res 55:79–88

Oświadczenia współautorów publikacji **Kołodziejczyk I., Bałabusta M., Szewczyk R., Posmyk M.M. (2015)** The levels of melatonin and its metabolites in conditioned corn (*Zea mays* L.) and cucumber (*Cucumis sativus* L.) seeds during storage. *Acta Physiologiae Plantarum* 37(6): 105 DOI:10.1007/s11738-015-1850-7 – wchodzącej w skład rozprawy doktorskiej:

| Imię i nazwisko | Szacunkowy udział [%] | Opis działań | Podpis |
|---|---|---|---|
| mgr Izabela Kołodziejczyk | 45 | Wykonanie eksperymentów dotyczących *Zea mays* L. Przygotowanie materiału roślinnego – dobór warunków i wykonanie hydrokondycjonowania (H, HMel50, HMel500). Przygotowanie ekstraktów do analiz HPLC-MS/MS jakościowych i ilościowych. Roczne kolekcjonowanie danych niezbędnych do analiz porównawczych. Opracowanie wyników dotyczących kukurydzy. Kompleksowa analiza i interpretacja wyników. Redakcja manuskryptu. | Kołodziejczyk Izabela |
| dr Marta Bałabusta* | 30 | Wykonanie eksperymentów dotyczących *Cucumis sativus* L. Przygotowanie materiału roślinnego – dobór warunków i wykonanie osmokondycjonowania (O, OMel50, OMel500). Przygotowanie ekstraktów do analiz HPLC-MS/MS, jakościowych i ilościowych. Roczne kolekcjonowanie danych niezbędnych do analiz. Opracowanie wyników dotyczących ogórka. | KIEROWNIK Katedry Ekofizjologii Roślin UŁ prof. dr hab. Małgorzata M. Posmyk |
| dr Rafał Szewczyk | 15 | Oznaczenie melatoniny i ej metabolitów metodą HPLC-MS/MS – konsultacja metodyczna. | |
| prof. dr hab. Małgorzata M. Posmyk | 10 | Współtwórca koncepcji pracy. Analiza statystyczna uzyskanych danych. Pomoc w interpretacji wyników i redakcji manuskryptu. | |
| Dane biblio-metryczne z dn. 19.05.2021 r. wg. *Web.Sci.*: **IF 1.563**$^{2015}$, **2.078**$^{V}$; **17** cytowań. (**70**$^{A}$ pkt-y MEiN) | | | |

** Z uwagi na brak kontaktu ze współautorką (dawną doktorantką KER) informacje na temat jej udziału w powstaniu pracy – zgodne ze stanem faktycznym i informacjami podanymi na końcu publikacji – certyfikuje promotor i zarazem kierownik Katedry, w której publikacja powstała.*

*Załącznik 3*

Acta Physiol Plant (2016) 38:146
DOI 10.1007/s11738-016-2166-y

ORIGINAL ARTICLE

# Exogenous melatonin expediently modifies proteome of maize (*Zea mays* L.) embryo during seed germination

**Izabela Kołodziejczyk[1] · Katarzyna Dzitko[2] · Rafał Szewczyk[3] · Małgorzata M. Posmyk[1]**

Received: 26 November 2015 / Revised: 6 May 2016 / Accepted: 9 May 2016


**Abstract** Melatonin (*N*-acetyl-5-methoxytryptamine) has a great potential for plant biostimulation. Its role in plant physiology is intensively explored, and its important function in plant stress defence frequently underlined. Melatonin is particularly effective when applied as an additional factor of seed priming. In the presented research, hydroconditioning was chosen experimentally as optimal for maize (*Zea mays* L.) seeds. The following seed variants were compared: controlled non-treated, hydroprimed with water and hydroprimed with melatonin solutions 50 and 500 μM. To identify modifications in proteome of maize seeds caused by the applied hydroconditioning techniques, protein extracts of germinated seed embryos (24 h, 25 °C) were separated by 2D-PAGE. Next, obtained maps of proteomes were compared (statistically and graphically) using PDQuest software, and characteristic spots of proteins were analysed qualitatively by mass-spectrometric techniques and identified in the Mascot protein databases. Research helped to identify hydropriming-associated proteins, and for the first time those which were expressed only in the presence of melatonin. Study confirmed that suitably selected pre-sowing treatment with melatonin, by embryo proteome modification, effectively prepares plants to adverse environmental conditions. In melatonin treated seeds during the initial state of embryos growth, even under optimal conditions, additional antioxidative, detoxifying, anti-stresses proteins were synthesized. Moreover, the supply of energy from seed storage substances was pretty intensified. The presented results partially explain how melatonin acts in plant stress defence, and why plants with higher melatonin content have exhibited increased capacity for stress tolerance.

**Keywords** Germination · Melatonin · Maize · Proteomics · Seed conditioning · *Zea mays*

Communicated by M Hajduch.

✉ Małgorzata M. Posmyk
posmyk@biol.uni.lodz.pl

[1] Department of Ecophysiology and Plant Development, Faculty of Biology and Environmental Protection, University of Lodz, 12/16 Banacha Str., 90-237 Lodz, Poland

[2] Department of Immunology and Infectious Biology, Faculty of Biology and Environmental Protection, University of Lodz, 12/16 Banacha Str., 90-237 Lodz, Poland

[3] Department of Industrial Microbiology and Biotechnology, Faculty of Biology and Environmental Protection, University of Lodz, 12/16 Banacha Str., 90-237 Lodz, Poland

## Introduction

Problems related to seed quality, their germination, seedling emergence, and development under various environmental stresses firmly influence on crop establishment and prospective yield. Seed priming/conditioning has become a common practice to obtain optimal seed performance and ultimate yield notably under adverse germination conditions (Jisha et al. 2013; Mabhaudhi and Modi 2011). Conditioning is defined as pre-sowing treatment in water that, by controlled seed hydration, leads to limited imbibition but prevents the emergence of radicle (Bradford and Bewley 2002). Seeds after priming can be redried to the initial moisture content and stored. Such conditioning can accelerate seed germination, improve seedling uniformity, and increase yields in various crops (Eskandari 2013; Rahimi, 2013). Among various conditioning techniques, e.g., osmopriming, matrix priming and hydropriming—the latter one is simple and low cost and, therefore, extremely

Published online: 19 May 2016

welcomed by farmers. Water pre-sowing treatments give the opportunity to apply biostimulators beneficial for plant. Biostimulators are different harmless substances mostly of natural origin that are able to improve plant development and stimulate life processes but in a different way than phytohormones and/or fertilizers. Their influence on plants is not the consequence of their direct ability to regulate metabolism, and their action could be multidirectional. The crucial point is that biostimulators in contrast to hormones improve plant metabolic processes without changing their natural pathway. They can facilitate the uptake of nutrients, stimulate root growth, also contribute to higher yield, and improve its quality via increase plants resistance/tolerance to unfavourable conditions, such as drought, extreme temperature, toxic pollutions, and so on (Basak 2008). Some studies indicate such a beneficial role of various substances, e.g. glycinebetaine, proline, natural nitrophenols (Asashi SL) as supplementation of irrigation into roots, with foliar fertilizers or protection spraying and also as seed treatment. Our previous research showed efficient conditioning methods for cucumber, cabbage and corn seed quality amelioration, with simultaneous melatonin application (Janas et al. 2009a; Posmyk et al. 2008, 2009a, b).

In general, results of our team suggest that melatonin (*N*-acetyl-5-methoxytryptamine) has a significant potential for biostimulation of plants (Janas and Posmyk 2013). Moreover, it is of natural origin, biodegradable, and harmless. Currently, the role of melatonin in plant physiology is being widely investigated. Solid evidence indicates melatonin as a growth promoter and plant development factor (Arnao and Hernández-Ruiz 2007; Chen et al. 2009; Hernández-Ruiz et al. 2005; Hernández-Ruiz and Arnao 2008; Sarrou et al. 2014; Zhang et al. 2014). The pre-sowing seed conditioning with melatonin protected cabbage seedlings against toxic effects of $Cu^{2+}$ (Posmyk et al. 2008). Moreover, melatonin application into maize and cucumber seeds had a positive effect on seedling development and yield of plants that grown from them, especially those subjected to water-stress (Zhang et al. 2013) or cold (Posmyk et al. 2009a, b).

The physiological effects of seed conditioning have been extensively studied in a wide range of crops (Chen and Arora 2013; Di Girolamo and Barbanti 2012). However, until now, the performance of primed seeds can only be verified by germination tests, which provide retrospective indication of the conditioning affects (Gallardo et al. 2001). Therefore, it is necessary to develop molecular markers improved vigour of primed seeds for commercial seed lots. Proteomics become widely applicable in seed research as a novel tool for protein characterisation and function analysis (Catusse et al. 2011; Gallardo et al. 2001; Wu et al. 2011). Seeds develop differently in dicots and monocots, especially the changes induced by priming treatment. Therefore, the results obtained from *Arabidopsis* may not be applicable in monocot crops (e.g., maize). To date, the proteomic analysis of protein profiles of seed priming in maize (*Zea mays* L.)—one of the most important crops widely planted in the world—has not been done yet. There is lack of data concerning effects of melatonin applied by hydropriming on protein profiles in germinating seeds.

This study aimed to analyse the hydropriming-induced modifications in corn embryo proteome and to indicate hydropriming-associated and melatonin-associated proteins in corn seeds. Comparative proteomic analysis was applied to identify differentially expressed proteins in the untreated and hydroconditioned seeds and in those hydroprimed with melatonin. The possible roles of indicated proteins in maize seed vigour were discussed.

## Materials and methods

### Plant material

Grains of *Zea mays* L. var. Ambrozja were provided by TORSEED (Torun, Poland). Before experiments, seeds were stored at room temperature, in darkness, under dry conditions, in tightly closed boxes.

### Hydropriming

Hydropriming was conducted by corn grains imbibing at 25 °C for 3 h, using distilled water or melatonin water solutions (50 or 500 μM). During conditioning seed, moisture content was increased from an initial 8.8 % (±0.2) to final 36.8 % (±2.3). The water quantity needed to enhance seed humidity was established experimentally. After soaking, seeds were redrying during 3 days at room temperature (20–23 °C; 40 ± 5 % RH) to obtain initial water content. Maize seed variants: non-treated (nt—gel A), hydroprimed (H—gel B), hydroprimed with melatonin 50, and 500 μM (HMel50—gel C and HMel500—gel D, respectively).

### Seed germination

All investigated seed variants were germinated in darkness at 25 °C for 24 h. Afterwards, axes were isolated, treated with liquid nitrogen, and then lyophilized.

### Protein preparation

Lyophilized tissue (200 mg) was homogenised (in ice bath) with 1.5 ml of extraction buffer 50-mM TrisHCl (pH 7.7) containing: 0.5-mM deoxycholate sodium salt (DOXC);

10-mM DL-dithiothreitol (DDT); and 10-mM EDTA; 1-mM phenylmethanesulfonylfluoride (PMSF). The homogenate was centrifuged: 20,000*g*/5 °C/10 min. The pellet was discarded, whereas proteins resuspended in the collected supernatant were precipitated with 0.07 % mercaptoethanol and 10 % trichloroacetic acid (TCA) in acetone, at 0 °C, during 45 min. The mixture was centrifuged at 20,000*g* for 10 min at 5 °C. The obtained pellet with precipitated proteins was then washed twice with 0.07 % mercaptoethanol in acetone. After the final centrifugation: 3000*g*/5 °C/5 min, sediment was vacuum-dried and then rediluted in buffer of Rabilloud consisting of: 65-mM DTT, 4 % CHAPS and 8 M urea. The proteins content was estimated in 96 microplates by Bradford (1976) method. The protein extracts were stored at −20 °C and then used for 2D PAGE.

## 2-D protein separation and image analysis

Protein separation and analysis was performed according to Kolodziejczyk et al. 2016. Samples of 300-mg proteins were applied to 11-cm IPG strips (pH 3–10, non-linear gradient ReadyStrip) in a Protean IEF cell (Bio-Rad, Hercules, CA) and in-gel rehydrated for 12 h. According to the isoelectric point, proteins were separated in the IEF-SYS (Scie-Plas Ltd. GB). Isoelectric focusing (first dimension separation) was performed at 18 °C for 72 h at 0–3000 V gradually changing (first 24 h: 0–1500 V; second: 1500–3000; third 3000 V). Before the second dimension PAGE, the gel strips were equilibrated twice with Eq solution (Eq: 50-mM Tris–HCl, pH 8.5, 6 M urea, 30 % glycerol, 10 % SDS) containing 1 % DTT for 15 min and next with Eq solution containing 2.5 % iodoacetamide. Then strips were applied to SDS–PAGE (4 % stacking gel and 12 % analytical gel) and fixed with 0.5 % agarose in 0.5 M Tris–HCl buffer (pH 6.8) and 10 % SDS. Appropriate protein markers (Thermo Scientific Page-Ruler Plus Prestained Protein Ladder No 26619) were loaded beside the strip. PAGE was performed in TV400YK Cooled Twin-Plate Maxi-Gel Electrophoresis Unit (Scie-Plas Ltd. GB) with SDS electrophoresis buffer containing: 25-mM Tris base, 192-mM glycine, 0.1 % SDS; for 2 h at 100 V and next for 2 h at 200 V, at constant temperature 25 °C. At least four independent biological replicates were performed to ensure the reliability of the results.

Separated proteins were visualised in gels using Imperial™ Protein Stain (Pierce). Then, stained gels were scanned at 300-dpi resolution using an Epson Perfection V700 Photo scanner. Obtained images were analysed with the PDQuest version 8.0.1 software (BioRad). Sample quantification was performed with four analytical gels originating from independent biological replicas. The volume of each spot was normalised to a relative volume, and mean values calculated from repeated data were compared. Qualitative and quantitative analysis sets were prepared. The cutoff for differentially expressed proteins was a fivefold change.

## Protein digestion

Indicated protein spots were cut out from stained gels and subjected to in-gel tryptic (Promega, Madison, WI) digestion, as it was described previously by Kołodziejczyk et al. 2016.

Every gel piece containing the protein of interest was first decolorized in solution: 50-mM $NH_4HCO_3$/acetonitrile (ACN) (50:50, v/v) for 15 min at room temperature. The colourless gel pieces were washed with ACN to dehydrate the band pieces. For reducing and alkylation, gel pieces were then washed with 10-mM DTT in 100-mM $NH_4HCO_3$ and incubate at 56 °C for 30 min, then with 50-mM iodoacetamide in 100-mM $NH_4HCO_3$ and incubate at room temperature for 30 min in dark. Then, the pieces were washed twice alternately with 100-mM $NH_4HCO_3$ and ACN. Finally, the band pieces were dried at room temperature and rehydrated overnight at 37 °C in 25-mM $NH_4HCO_3$ with trypsin (Promega). After this digestion, gel bands were extracted twice with 2 % ACN/0.1 % trifluoroacetic acid (TFA) to collect the remaining peptides. The obtained supernatant was used for LC–MS/MS analysis.

## LC–MS/MS analysis

LC–MS/MS analysis was performed on Eksigent Express HT Micro LC and AB Sciex QTRAP 3200 mass spectrometer with a microspray ESI ion source installed, as it was described by Kołodziejczyk et al. 2016.

Samples were ultrasonicated for 30 s, shaken on vortex, and centrifuged for 5 min. 10 µl of the protein sample was applied by injection mode directly on Eksigent C8-CL-120 column (3 µm 120 Å, 0.5 × 100 mm) at 40 °C. The mobile phase was: water with 0.1 % formic acid (A) and ACN with 0.1 % formic acid (B). Gradient profile (10 µl/min constant flow conditions) started from 95 % A for 1 min, followed by 60 % A after 40 min and 5 % in 45 min and maintained until 50 min of the run. The column equilibration under the initial gradient conditions was applied from 50.1 to 53 min and as a prerun for 1 min before subsequent injection. MS/MS detection was performed using information-dependent acquisition method (IDA) composed of mixed positive ionization scan modes and IDA criteria for dynamic *m*/*z* filtering. The process was constructed as follows: enhanced MS scan (EMS), enhanced resolution scan (ER), IDA criteria and enhanced product ion scan (EPI). Overall survey of EMS scan parameters comprehended the mass range 500–1400 Da

with scan speed 4000 Da/s and 50–70 V declustering potential (DP) values. ER scan was active in 250 Da/s scan rate (DP = 50–70 V) and was used for peptide charge determination. EPI scan was active in the range 50–1600 *m/z* (DP = 60 V and optimised rolling collision energy) and was applied for mass spectra collection and for the peptide sequence analysis. The ion source parameters were: CUR: 25.00; TEM: 400.00; GS1: 20.00; GS2: 40.00; ihe: ON; IS: 5000.00; CAD: high. The most important IDA criteria applied for selective *m/z* filtering were as follows: dynamic background subtraction—on, choose 1–3 most intense peaks from range 500–1400 *m/z* which charge state is 2–4 (include unknown) and exceeds 10,000 counts intensity, exclude former target ions for 30 s after 3 occurrences and use enhanced resolution scan to confirm charge state.

### Database searching

The ProteinPilot v 4.0.8 software (AB Sciex, USA) and the MASCOT search engine v. 2.3 were applied for the database searching, as those were described previously by Kołodziejczyk et al. 2016.

The data were searched against the *Zea mays* (over 212,000 sequences) database extracted from NCBI (version 05.2013). Mascot MS/MS ion searches were conducted using trypsin as the digesting enzyme, up to two missed cleavages were tolerated, and the following modifications were applied: acetyl (N-term), carbamidomethyl (C), carbamyl (N-term), deamidated (NQ), formyl (N-term), Gln to pyro-Glu (N-term Q), Glu to pyro-Glu (N-term E) and oxidation (M). The searches were performed with a peptide mass tolerance of 0.7 Da and fragment ion mass tolerance of 0.3 Da. The proteins established by MASCOT searches were further processed using a BLAST search against the NCBI non-redundant protein sequence database (*circa* 39 million sequences) applying the algorithm of domain enhanced lookup time accelerated BLAST (DELTA-BLAST) to confirm and/or define the probable protein function.

## Results and discussion

Positive effects of conditioning were not visible in corn seed germination tests performed under optimal temperature conditions. Except that hydroprimed seeds germinated more uniformly (smaller ± SEM), both the germination rate and the final germination percentage were comparable in good quality control seeds and their primed equivalents (Janas et al. 2009a). However, subsequent experiments showed that the seedlings grown from the seeds hydroconditioned with melatonin extremely well-tolerated stresses of suboptimal temperature (10 °C), and heavy metal contamination (2.5 mM $Cu^{2+}$) and also regenerated much better after relief of stress. It was manifested by better growth (greater weight of seedlings) and higher chlorophyll content and phenolic synthesis in the seedlings developed from the seeds hydroprimed with melatonin (Janas et al. 2009a). These experiments of our team provoked reflection that if the effects of conditioning are not visible in seed parameters investigated under the optimal conditions maybe the explanation why the seedlings grown from the seeds pretreated with melatonin tolerate stresses so well can be found at the molecular/proteomic level.

That is why proteomes of embryos isolated from particular seed variants (nt, H, HMel50 and, HMel500) germinated 24 h at optimal temperature (25 °C) were compared. The seed variants: nt and H, were the reference for the detection of melatonin-associated proteins, since it is well known that already the conditioning with water provokes modifications in seed proteome (Catusse et al. 2011; Wu et al. 2011; Gallardo et al. 2001).

The protein maps made in four replicates pointed out a significant level of reproducibility. Specialised software was able to indicate as corresponding ~95 % of the stained protein spots in the replicated gel images. PDQuest densitometric analysis of master gels was principle for qualitative (Table 1) and quantitative analyses of particular experimental seed variants (Tables 2, 3). 130 protein spots were indicated on gel A—representing proteome of axes isolated from nt seed variant, 143 on gel B—axes proteome from H seeds, 180 on gel C—axes proteome from HMel50 seeds, and 160 on gel D—axes proteome from HMel500 seeds (Figs. 1, 2). Our data suggested that hydroconditioning and especially hydroconditioning supplemented with melatonin induced new proteins biosynthesis in grain embryonic axes (Fig. 2). Previous comparative proteomic analysis by Gong et al. (2013) identified eight protein spots, which markedly differed in abundance between the primed and unprimed maize seeds, while the abundance of approximately 98 % spots was almost the same. However, the mentioned study was performed with dry—no imbibed/germinated primed and unprimed seeds. Thus, during the short hydropriming process, metabolic activities (such as protein synthesis and degradation) were quite low. We performed our study after first 24 h of germination under optimal conditions, when the metabolic processes were very intensive. Moreover, we used the relatively sensitive and effective Imperial™ Protein Stain (Pierce)—in comparison with Coomassie Brilliant Blue or Silver Stain—to visualise proteins in 2-DE gels; thereby, even the changes of low-abundance proteins might be detected.

**Table 1** Identification of the proteins unique to different seed variants: control, non-treated (A), hydroprimed (B), all primed (B, C, D), all hydroprimed with melatonin (C, D) and characteristic of the seeds treated with 50-μM (C) or 500-μM (D) melatonin, determined after 24 h of germination at optimal 25 °C

| Spot No | Function category | Protein family | Protein name | Accession No | Mascot score | % Cover | Peptide +/− | MW [kDa] | p*I* |
|---|---|---|---|---|---|---|---|---|---|
| **Protein unique to control, non-primed seeds (1)—A** | | | | | | | | | |
| *Protein destination and storage* | | | | | | | | | |
| 0402 | 06.13 | Phytepsin | Aspartic proteinase oryzasin-1 precursor [*Zea mays*] | gil226506070 | 65 | 10 | 3/1 | 54.90 | 5.41 |
| **Protein unique to hydroprimed seeds (0)—B** | | | | | | | | | |
| **Proteins unique to all primed seeds (14)—B, C, D** | | | | | | | | | |
| *Metabolism* | | | | | | | | | |
| 4503 | 01.01 | Trp-synth-beta II | Cysteine synthase precursor [*Zea mays*] | gil295421203 | 321 | 40 | 13/7 | 34.19 | 5.91 |
| 1601 | 01.03 | Ribokinase pfkB | Adenosine kinase [*Zea mays*] | gil4582787 | 332 | 29 | 13/6 | 36.01 | 5.23 |
| 5301 | 01.04 | Pyrophosphatase | Inorganic pyrophosphatase [*Zea mays*] | gil414586181 | 59 | 5 | 1/1 | 24.35 | 5.46 |
| *Energy* | | | | | | | | | |
| 5602 | 02.01 02 | Gp dh N | Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic isoform X1 [*Zea mays*] | gil6016075 | 122 | 16 | 3/2 | 31.97 | 6.4 |
| 7502 | 02.01 02 | Gp dh N | Cytosolic glyceroldehyde-3-phosphate dehydrogenase GAPC3 [*Zea mays*] | gil6166167 | 76 | 18 | 5/1 | 36.43 | 7.01 |
| 2301 | 02.07 | SugarP isomerase | 6-phosphogluconolactonase isoform 1 [*Zea mays*] | gil414591367 | 98 | 22 | 7/3 | 34.76 | 7.71 |
| *Protein destination and storage* | | | | | | | | | |
| 1801 | 06.01 | PDIb | Protein disulphide isomerase (PDI) [*Zea mays*] | gil145666464 | 447 | 33 | 25/12 | 56.7 | 5.01 |
| 2802 | 06.01 | Chaperonin like | Chaperonin 60 [*Zea mays*] | gil257734900 | 79 | 9 | 4/1 | 61.2 | 5.68 |
| 8103 | 06.20 | Cupin 2 | Cupin family protein [*Zea mays*] | gil226509468 | 68 | 11 | 4/1 | 56.49 | 6.1 |
| *Disease/defence* | | | | | | | | | |
| 3502 | 11.05 | SDR | General stress protein 39 [*Zea mays*] | gil414590804 | 79 | 14 | 5/1 | 38.6 | 9.06 |
| 4502 | 11.05 | SDR | General stress protein 39 [*Zea mays*] | gil195659117 | 709 | 47 | 29/17 | 32.92 | 5.78 |
| 3901 | 11.05 | HSP70 actin | Heat shock 70 kDa protein [*Zea mays*] | gil414589839 | 141 | 15 | 9/2 | 72.50 | 5.62 |
| 6902 | 11.05 | MDR | Alcohol dehydrogenase 1 isoform X1 [*Zea mays*] | gil7262819 | 89 | 5 | 2/2 | 40.96 | 6.28 |
| 6001 | 11.06 | Thioredoxin like | Thioredoxin homolog 2 protein [*Zea mays*] | gil66841004 | 61 | 8 | 1/1 | 13.03 | 6.19 |
| **Proteins unique to seeds hydroprimed with melatonin (14)—C, D** | | | | | | | | | |
| *Energy* | | | | | | | | | |
| 6502 | 02.01 02 | Gp dh C | Glyceraldehyde-3-phosphate dehydrogenase, partial [*Zea mays*] | gil6016075 | 182 | 12 | 4/2 | 24.93 | 8.44 |
| *Cell growth/division* | | | | | | | | | |
| 7804 | 03.22 | APP MetAP | Proliferation-associated protein 2G4 [*Zea mays*] | gil413945091 | 117 | 10 | 6/4 | 43.20 | 6.58 |
| *Transcryption* | | | | | | | | | |
| 2503 | 04.01 | Ribosomal L10 P0 | 60S acidic ribosomal protein P0 [*Zea mays*] | gil413941828 | 124 | 9 | 4/3 | 34.47 | 5.2 |
| *Protein synthesis* | | | | | | | | | |
| 0301 | 05.04 | EF1B | Elongation factor 1-delta 1 [*Zea mays*] | l414887578 | 199 | 22 | 6/4 | 24.84 | 4.39 |

**Table 1** continued

| Spot No | Function category | Protein family | Protein name | Accession No | Mascot score | % Cover | Peptide +/− | MW [kDa] | p*I* |
|---|---|---|---|---|---|---|---|---|---|
| *Protein destination and storage* | | | | | | | | | |
| 2604 | 06.07 | Aha1 N | Activator of 90 kDa heat shock protein ATPase [*Zea mays*] | gil414869818 | 89 | 6 | 2/2 | 38.58 | 5.33 |
| 2501 | 06.13 | Ntn hydrolases | Proteasome subunit alpha type 1 [*Zea mays*] | gil226531007 | 277 | 46 | 11/7 | 30 | 5,19 |
| 3202 | 06.20 | Cupin 2 | Cupin family protein [*Zea mays*] | gil4139567 | 67 | 4 | 2/1 | 71 | 6.30 |
| 4301 | 06.20 | Cupin 2 | Globulin-1 S allele-like [*Zea mays*] | gil413956703 | 255 | 10 | 6/5 | 71.09 | 6.31 |
| 7401 | 06.20 | Cupin 2 | Globulin-1 S allele precursor [*Zea mays*] | gil195658011 | 136 | 11 | 5/2 | 49.93 | 6.16 |
| *Transporters* | | | | | | | | | |
| 2801 | 07.25 | ABC ATPase | ATP synthase subunit beta family protein [*Zea mays*] | gil414880947 | 828 | 50 | 33/22 | 59.28 | 5.95 |
| *Disease/defence* | | | | | | | | | |
| 3105 | 11.05 | α -crystallin-Hsps | Heat shock 22 kDa protein [*Zea mays*] | gil195621504 | 104 | 16 | 3/2 | 23.85 | 6.47 |
| 7102 | 11.05 | α-crystallin-Hsps | Class II heat shock protein [*Zea mays*] | gil413939226 | 208 | 39 | 9/6 | 18.34 | 6.6 |
| 0203 | 11.06 | PITH | Thioredoxin family Trp26 isoform 4 [*Zea mays*] | gil414881868 | 177 | 27 | 6/4 | 22.60 | 4.95 |
| *Secondary metabolism* | | | | | | | | | |
| 8506 | 20.01 | DAHP synth1 | 2-dehydro-3-deoxyphosphooctonate aldolase [*Zea mays*] | gil414878308 | 64 | 8 | 1/1 | 15.89 | 5.07 |
| **Proteins unique to seeds hydroprimed with 50 µM melatonin (23)—C** | | | | | | | | | |
| *Energy* | | | | | | | | | |
| 1501 | 02.01 | Bac FRK | Fructokinase 1 [*Zea mays*] | gil75293604 | 152 | 28 | 6/3 | 34.67 | 4.87 |
| 4805 | 02.01 | alkPPc | 2,3-bisphosphoglycerate-independent phosphoglycerate mutase isoform XI [*Zea mays*] | gil551288 | 118 | 10 | 6/3 | 60.58 | 5.29 |
| 3805 | 02.01 | TIM phosphate binding | Triosephosphate isomerase, cytosolic [*Zea mays*] | gil257353728 | 64 | 10 | 3/1 | 60.50 | 5.34 |
| 7701 | 02.01 | TIM phosphate binding | Aldolase1 [*Zea mays*] Fructose-bisphosphate aldolase cytoplasmic isozyme [*Zea mays*] | gil414879138 | 419 | 35 | 15/10 | 38.57 | 7.52 |
| 7603 | 02.01 02 | Gp dh N | Glyceraldehyde-3-phosphate dehydrogenase1 isoform 1 [*Zea mays*] | gil413921396 | 351 | 45 | 10/7 | 31.92 | 6.45 |
| 7702 | 02.01 02 | Gp dh N | Glyceraldehyde-3-phosphate dehydrogenase1 cytosolic isoform X1 [*Zea mays*] | gil413921395 | 115 | 13 | 3/1 | 36.47 | 6.46 |
| 2602 | 02.10 | CoA-ligase | Succinyl-CoA ligase beta-chain [*Zea mays*] | gil226510248 | 311 | 29 | 15/8 | 45.17 | 5.99 |
| 5504 | 02.10 | SDR | Malate dehydrogenase [*Zea mays*] | gil550576396 | 119 | 9 | 3/2 | 35.62 | 7.63 |
| *Cell growth/division* | | | | | | | | | |
| 2401 | 03.01 | SMP, LEA | Late embryogeneis abundant protein D-34 [*Zea mays*] | gil414872767 | 226 | 17 | 4/4 | 27.16 | 5.41 |
| 0701 | 03.19 | UBQ | DNA repair protein RAD23 [*Zea mays*] | gil525344153 | 62 | 2 | 1/1 | 42.57 | 4.61 |
| *Protein synthesis* | | | | | | | | | |
| 0303 | 05.04 | EF1B | Elongation factor 1-beta [*Zea mays*] | gil414887997 | 141 | 26 | 6/2 | 23.4 | 4.55 |

**Table 1** continued

| Spot No | Function category | Protein family | Protein name | Accession No | Mascot score | % Cover | Peptide +/− | MW [kDa] | p*I* |
|---|---|---|---|---|---|---|---|---|---|
| *Protein destination and storage* | | | | | | | | | |
| 0002 | 06.01 | BBI plant serine protease inhibitors | Hypothetical protein ZEAMMB73_733594 [*Zea mays*] 66 % identities to Bowman-Birk trypsin inhibitor TI1 [*Coix lacryma-jobi*] | gil414876537 | 54 | 4 | 1/1 | 26.05 | 5.45 |
| 6301 | 06.01 | SPFH like | Prohibitin2 [*Zea mays*] | gil7716458 | 79 | 13 | 2/2 | 30.70 | 6.55 |
| 8401 | 06.13 | Ntn hydrolases | 20S proteasome alpha 4 subunit [*Zea mays*] | gil414590073 | 201 | 18 | 6/4 | 27.21 | 8.3 |
| *Cell structure* | | | | | | | | | |
| 2001 | 09.04 | ADF gelsolin | Actin-depolymerizing factor 3 [*Zea mays*] | gil413932602 | 258 | 65 | 10/6 | 15.9 | 5.47 |
| 8101 | 09.07 | C2 | Unnamed protein product [*Zea mays*] 98 % identities to ADP-ribosylation factor GTPase-activating protein AGD11 [*Zea mays*] | gil257692306 | 62 | 12 | 1/1 | 18.92 | 6.59 |
| *Disease/defence* | | | | | | | | | |
| 0201 | 11.05 | α-crystallin-Hsps | Co-chaperone protein SBA1 [*Zea mays*] | gil413920965 | 50 | 3 | 1/1 | 20.80 | 4.43 |
| 0202 | 11.05 | TCTP | Translationally controlled tumour protein homolog [*Zea mays*] | gil413935193 | 69 | 16 | 4/1 | 18.68 | 4.52 |
| 0501 | 11.05 | USP | USP family protein [*Zea mays*] | gil413952234 | 81 | 6 | 1/1 | 27.12 | 4.88 |
| 2601 | 11.05 | GAT 1 | Hypothetical protein ZEAMMB73_682876 [*Zea mays*] 88 % identities to Protein DJ-1 homolog B-like [*Setaria italic*] | gil413954138 | 195 | 13 | 7/4 | 42.22 | 5.51 |
| 2402 | 11.06 | Glo EDI BRP like | Glyoxalase I [*Zea mays*] | gil37932483 | 120 | 23 | 7/3 | 35.14 | 6.62 |
| 6201 | 11.06 | Thioredoxin like | 1-Cys peroxiredoxin antioxidant PER1 [*Zea mays*] | gil414887819 | 165 | 28 | 6/3 | 24.96 | 6.38 |
| 6402 | 11.06 | Esterase lipase | Esterase D [*Zea mays*] | gil413951351 | 121 | 10 | 2/2 | 31.87 | 6.13 |
| **Proteins unique to seeds hydroprimed with 500 µM melatonin (3)—D** | | | | | | | | | |
| *Metabolism* | | | | | | | | | |
| 0901 | 01. … | Transferases | Transferase [*Zea mays*] | gil126633162 | 62 | 4 | 2/1 | 75.4 | 5.16 |
| 5803 | 01.03 | TIM phosphate binding | Inosine-5-monophosphate dehydrogenase 2 [*Zea mays*] | gil226507304 | 81 | 11 | 4/2 | 52.65 | 5.89 |
| *Protein synthesis* | | | | | | | | | |
| 5603 | 05.04 | ABC ATPase | Translation elongation/initiation factor family protein [*Zea mays*] | gil413932419 | 138 | 19 | 6/2 | 43.84 | 5.76 |

The table contains: spots excised from gels and numbered by PDQuest Software used for gel qualitative analysis; function category consistent with *Nature* (Bevan et al. 1998); protein family's, names and accession numbers consistent with NCBI database; Mascot score, sequence coverage (%), number of matched/unmatched (+/−) peptides, MW [kDa] and p*I* values

## Influence of hydropriming on axes proteome in germinated maize grains

Proteomic analysis of extracts from particular seed variants (nt, H, HMel50 and HMel500) enabled to identify 14 proteins, which appeared after seed conditioning (presented in gels B, C, and D—Table 1; Fig. 1BCD).

There were the proteins related to: (1) cysteine biosynthesis (cysteine synthase precursor—4503)—an endogenic sulphuric amino acid with –SH group and by glutathione metabolism connected/associated with the detoxification processes and regulation of cell redox status; (2) the preservation of intracellular adenylate pools due to regulation of extracellular adenosine levels (adenosine kinase—

**Table 2** Proteins showing different expressions in axes of the control nt seeds (A) germinated for 24 h at optimal 25 °C, to those from all hydroprimed seeds: H, HMel50, HMel500 (B, C, D)

| Spot No | Function category | Protein name | Accession No | Mascot score | % Cover | Peptides +/− | MW [kDa] | p*I* | Fold change $p \leq 0.01$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | A/B | A/C | A/D |
| **Proteins up-regulated in A in comparison with B, C, D** | | | | | | | | | | | |
| *Metabolism* | | | | | | | | | | | |
| 1301 | 01.04 | Adenylate cyclase [*Zea mays*] | gil414888163 | 57 | 8 | 3/0 | 23.51 | 5.18 | **4.99** | 1.14 | **5.45** |
| *Energy* | | | | | | | | | | | |
| 5601 | 02.10 | Malate dehydrogenase, cytoplasmic [*Zea mays*] | gil320449084 | 498 | 43 | 16/12 | 35.50 | 5.76 | 3.04 | **19.3** | 2.65 |
| *Cell growth/division* | | | | | | | | | | | |
| 8803 | 03.01 | Embryonic protein DC-8-like [*Zea mays*] | gil413936531 | 107 | 5 | 2/2 | 61.73 | 7.27 | **34.1** | **18.6** | |
| *Protein synthesis* | | | | | | | | | | | |
| 9701 | 05.04 | Elongation factor 1 alpha [*Zea mays*] | gil7230387 | 162 | 14 | 7/3 | 49.12 | 9.2 | 1.8 | 7.6 | |
| *Protein destination and storage* | | | | | | | | | | | |
| 2903 | 06.01 | Mitochondrial chaperonin-60 [*Zea mays*] | gil22248 | 1342 | 47 | 49/33 | 61.15 | 5.68 | **40.8** | 2.71 | **5.63** |
| 4203 | 06.13 | Proteasome subunit beta type 6 precursor [*Zea mays*] | gil229612088 | 169 | 22 | 6/3 | 26.16 | 5.47 | **7.63** | 1.13 | 2.23 |
| 1702 | 06.13 | Hypothetical protein ZEAMMB73_715164 [*Zea mays*]<br>96 % identity to 26S protease regulatory subunit 6A homolog [*Oryza sativa* Japonica Group] | gil413944465 | 77 | 13 | 6/2 | 47.78 | 4.94 | **13.3** | 1.29 | 1.51 |
| *Cell structure* | | | | | | | | | | | |
| 2105 | 09.04 | Actin depolymerizing factor [*Zea mays*] | gil414873646 | 71 | 36 | 5/1 | 15.89 | 5.46 | 1.11 | 4.19 | **5.97** |
| *Disease/defence* | | | | | | | | | | | |
| 5502 | 11.05 | General stress protein 39 [*Zea mays*] | gil414590803 | 829 | 50 | 24/18 | 38.60 | 5.78 | 1.47 | **7.52** | 2.44 |
| 2901 | 11.05 | Unknown [*Zea mays*] 98 % identities to Heat shock 70 kDa protein, mitochondrial-like [*Setaria italica*] | gil194688822 | 289 | 17 | 11/7 | 72.62 | 5.54 | **8.62** | **6.37** | 3.77 |
| 1001 | 11.06 | Glyoxalase family protein superfamily [*Zea mays*] | gil195604212 | 314 | 42 | 8/5 | 15.07 | 5.47 | **76.6** | 1.98 | 1.98 |
| *Unclassified* | | | | | | | | | | | |
| 5402 | 13 | Secreted protein [*Zea mays*] | gil413951152 | 108 | 29 | 6/4 | 27.33 | 5.84 | **12.6** | **17.4** | |
| **Proteins down-regulated in A in comparison with B, C, D.** | | | | | | | | | | | |
| *Energy* | | | | | | | | | | | |
| 4201 | 02.01 | Triose-phosphate isomerase [*Zea mays*] (EC 5.3.1.1) | gil414876338 | 187 | 25 | 5/5 | 27.01 | 5.52 | | **0.11** | **0.10** |
| 4701 | 02.01 | Cytosolic 3-phosphoglycerate kinase [*Zea mays*] | gil413935730 | 405 | 20 | 13/12 | 60.80 | 9.41 | 6.85 | **0.15** | 0.22 |
| 8601 | 02.01 02 | Fructose-bisphosphate aldolase [*Zea mays*] | gil413951593 | 536 | 38 | 21/13 | 38.44 | 6.96 | **0.03** | **0.17** | **0.09** |
| 8604 | 02.01 02 | Fructose-bisphosphate aldolase, cytoplasmic isozyme [*Zea mays*] | gil113621 | 115 | 9 | 2/2 | 38.58 | 7.52 | **0.01** | 0.39 | **0.02** |

**Table 2** continued

| Spot No | Function category | Protein name | Accession No | Mascot score | % Cover | Peptides +/− | MW [kDa] | p*I* | Fold change $p \leq 0.01$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | A/B | A/C | A/D |
| 8602 | 02.10 | Succinyl-CoA ligase alpha-chain 2 [*Zea mays*] | gil414887289 | 61 | 12 | 3/0 | 34.19 | 8.16 | 0.31 | **0.02** | **0.08** |
| *Cell growth/division* | | | | | | | | | | | |
| 6602 | 03.01 | Hypothetical protein ZEAMMB73_579239 [*Zea mays*] 80 % identities to LEA protein [*Setaria italica*] | gil413955864 | 66 | 11 | 3/1 | 37.89 | 6.6 | **0.05** | **0.13** | **0.05** |
| *Transcryption* | | | | | | | | | | | |
| 8902 | 04.19 22 | Hypothetical protein ZEAMMB73_983793 [*Zea mays*] 94 % identities to Polyadenylate-binding protein 2-like [*Setaria italica*] | gil414588971 | 62 | 7 | 4/0 | 71.13 | 7.55 | 0.24 | 0.27 | 0.17 |
| *Protein destination and storage* | | | | | | | | | | | |
| 8202 | 06.01 | 1-Cys peroxiredoxin antioxidant [*Zea mays*] | gil87133468 | 115 | 10 | 4/2 | 24.89 | 6.31 | | **0.07** | **0.10** |
| 3101 | 06.02 | Globulin 2 [*Zea mays*] | gil414873301 | 291 | 14 | 8/6 | 49.89 | 6.16 | 0.21 | **0.03** | **0.15** |
| 6302 | 06.13 | Proteasome subunit alpha type [*Zea mays*] | gil414865138 | 504 | 49 | 17/13 | 27.40 | 6.10 | **0.03** | **0.11** | 1.17 |
| 5304 | 06.13 | Proteasome subunit alpha type [*Zea mays*] | gil414865138 | 330 | 39 | 13/9 | 27.4 | 6.1 | 0.42 | **0.02** | |
| 3702 | 06.20 | Homogentisate 1,2-dioxygenase [*Zea mays*] | gil413953601 | 149 | 7 | 7/3 | 50.30 | 5.30 | **0.19** | **0.17** | 0.39 |
| *Signal transduction* | | | | | | | | | | | |
| 6003 | 10.0404 | Nucleoside diphosphate kinase [*Zea mays*] | gil414867768 | 84 | 10 | 1/1 | 16.53 | 6.3 | **0.013** | **0.032** | **0.064** |
| 8001 | 10.0404 | Nucleoside diphosphate kinase 4 [*Zea mays*] | gil413946787 | 64 | 4 | 1/0 | 25.93 | 9.04 | **0.09** | **0.04** | **0.16** |
| *Disease/defence* | | | | | | | | | | | |
| 4601 | 11.02 | Protein Z [*Zea mays*] | gil195606550 | 141 | 16 | 10/5 | 42.16 | 5.52 | 1.12 | **0.08** | 0.36 |
| 5001 | 11.05 | 16.9 kDa class I heat shock protein 1 [*Zea mays*] | gil296512085 | 59 | 24 | 4/1 | 17.05 | 6.77 | **0.10** | 0.66 | |
| 7002 | 11.05 | Stress-inducible membrane pore protein [*Zea mays*] | gil195642018 | 146 | 40 | 5/3 | 17.80 | 6.41 | | 0.39 | **0.02** |
| 3301 | 11.05 | General stress protein 39 [*Zea mays*] | gil414590803 | 71 | 6 | 2/1 | 38.6 | 9.06 | **0.11** | **0.04** | |
| *Unclassified* | | | | | | | | | | | |
| 8003 | 13 | CBS domain protein isoform 3 [*Zea mays*] | gil414872657 | 359 | 40 | 11/8 | 22.48 | 9.35 | 0.37 | **0.11** | **0.11** |

The table contains: spots excised from gels and numbered by PDQuest Software used for quantitative gel analysis; function category consistent with *Nature* (Bevan et al. 1998); protein names and accession numbers consistent with NCBI database; Mascot score, sequence coverage (%), number of matched/unmatched (+/−) peptides, MW [kDa] and p*I* values; and relative abundance fold change in comparison with control nt (A) seeds ($p \leq 0.01$)—bolded number indicate ≥5-fold changes in protein expression in the compared variants

1601); and (3) phosphate metabolism (inorganic pyrophosphatase—5301).

In the axes of primed seeds, there were also a number of additional enzymes involved in cell energy metabolism: glyceraldehyde-3-phosphate dehydrogenase 1 cytosolic isoform X1—5602; cytosolic glyceraldehyde-3-phosphate dehydrogenase GAPC3—7502; 6-phosphogluconolactonase isoform 1—2301 (Table 1; Fig. 1BCD). These enzymes are involved in gluconeogenesis and glycolysis, also in the pentose phosphate pathway (the latter one). Improvement of the energy acquisition from sugar turnover is of utmost importance for growth and development of

**Table 3** Proteins showing different expressions in axes of the hydroprimed seeds (B) germinated 24 h at optimal 25 °C, to those from all seeds hydroprimed with melatonin: HMel50, HMel500 (C, D)

| Spot No | Function category | Protein name | Accession No | Mascot score | % Cover | Peptides +/− | MW [kDa] | p*I* | Fold change $p \leq 0.01$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | B/C | B/D |
| **Proteins up-regulated in B in comparison with C, D** | | | | | | | | | | |
| *Energy* | | | | | | | | | | |
| 8501 | 02.01 02 | Cytosolic glyceraldehyde-3-phosphate dehydrogenase [*Zea mays*] | gi\|6016075 | 640 | 61 | 22/11 | 37.16 | 6.67 | 4.32 | **6.62** |
| 8604 | 02.01 02 | Fructose-bisphosphate aldolase, cytoplasmic isozyme [*Zea mays*] | gi\|113621 | 115 | 9 | 2/2 | 38.58 | 7.52 | **36.4** | 1.60 |
| *Protein destination and storage* | | | | | | | | | | |
| 8103 | 06.20 | Cupin family protein [*Zea mays*] | gi\|195606798 | 68 | 11 | 4/1 | 56.49 | 6.1 | 4.16 | **11.1** |
| *Cell structure* | | | | | | | | | | |
| 2105 | 09.04 | Actin depolymerizing factor [*Zea mays*] | gi\|414873646 | 71 | 36 | 5/1 | 15.89 | 5.46 | 3.76 | **5.36** |
| *Signal transduction* | | | | | | | | | | |
| 6003 | 10.0404 | Nucleoside diphosphate kinase [*Zea mays*] | gi\|414867768 | 84 | 10 | 1/1 | 16.53 | 6.3 | 2.57 | **5.09** |
| 5503 | 10.0410 | Guanine nucleotide-binding protein β-subunit-like [*Zea mays*] | gi\|413948362 | 176 | 12 | 5/4 | 36.21 | 6.13 | **9.14** | 0.88 |
| *Disease/defence* | | | | | | | | | | |
| 1202 | 11.05 | Heat shock protein 26 [*Zea mays*] | gi\|453670 | 84 | 22 | 4/1 | 26.36 | 7.88 | 3.70 | **60.8** |
| 5202 | 11.06 | 1-Cys peroxiredoxin antioxidant [*Zea mays*] | gi\|87133468 | 204 | 29 | 7/4 | 24.96 | 6.38 | **5.07** | 0.72 |
| 5502 | 11.05 | General stress protein 39 SDR family protein [*Zea mays*] | gi\|414590803 | 829 | 50 | 24/18 | 38.60 | 5.78 | **5.11** | 1.66 |
| 6001 | 11.06 | Thioredoxin h2 protein [*Zea mays*] | gi\|66841004 | 61 | 8 | 1/1 | 13.03 | 6.19 | 2.31 | **8.09** |
| **Proteins down-regulated in B in comparison with C, D** | | | | | | | | | | |
| *Metabolism* | | | | | | | | | | |
| 1601 | 01.03 | Adenosine kinase [*Zea mays*] | gi\|4582787 | 332 | 29 | 13/6 | 36.01 | 5.23 | **0.08** | **0.08** |
| 1602 | 01.01 | Agmatine deiminase [*Zea mays*] | gi\|226508546 | 133 | 12 | 4/3 | 41.6 | 4.93 | **0.14** | **0.14** |
| 3701 | 01.05 | UTP-glucose-1-phosphate uridylyltransferase [*Zea mays*] | gi\|212275097 | 281 | 21 | 10/6 | 52.06 | 5.3 | **0.11** | 0.31 |
| 4503 | 01.01 02 | O-acetylserine (thiol) lyase [*Zea mays*] | gi\|758353 | 321 | 40 | 13/7 | 34.19 | 5.91 | 0.61 | **0.18** |
| *Energy* | | | | | | | | | | |
| 2301 | 02.07 | 6-phosphogluconolactonase isoform 2 [*Zea mays*] | gi\|414591367 | 98 | 22 | 7/3 | 34.76 | 7.71 | **0.14** | **0.07** |
| 2701 | 02.01 02 | Enolase [*Zea mays*] | gi\|22273 | 690 | 34 | 15/11 | 48.03 | 5.2 | **0.11** | |
| 4202 | 02.01 | Triosephosphate isomerase, cytosolic [*Zea mays*] | gi\|414876339 | 531 | 42 | 18/11 | 33.04 | 6.96 | **0.19** | **0.15** |
| 4602 | 02.10 | Malate dehydrogenase [*Zea mays*] | gi\|414871066 | 148 | 26 | 7/4 | 35.59 | 5.4 | 0.40 | **0.16** |
| 4701 | 02.01 | Cytosolic 3-phosphoglycerate kinase [*Zea mays*] | gi\|413935730 | 405 | 20 | 13/12 | 60.80 | 9.41 | **0.02** | **0.03** |
| 5602 | 02.01 02 | Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic [*Zea mays*] | gi\|6016075 | 122 | 16 | 3/2 | 31.97 | 6.4 | **0.17** | 0.56 |
| 8601 | 02.01 02 | Fructose-bisphosphate aldolase [*Zea mays*] | gi\|413951593 | 536 | 38 | 21/13 | 38.44 | 6.96 | **0.03** | **0.17** |
| 8602 | 02.10 | Succinyl-CoA ligase alpha-chain 2 [*Zea mays*] | gi\|414887289 | 51 | 12 | 3/0 | 34.19 | 8.16 | **0.07** | 0.24 |
| *Protein destination and storage* | | | | | | | | | | |
| 2802 | 06.01 | Chaperonin 60 [*Zea mays*] | gi\|309557 | 79 | 9 | 4/1 | 61.2 | 5.68 | 0.23 | **0.05** |
| 2903 | 06.01 | Mitochondrial chaperonin-60 [*Zea mays*] | gi\|22248 | 1342 | 47 | 49/33 | 61.15 | 5.68 | **0.07** | **0.14** |
| 3101 | 06.20 | Globulin 2 [*Zea mays*] | gi\|414873301 | 291 | 14 | 8/6 | 49.89 | 6.16 | **0.16** | 0.74 |
| 3103 | 06.20 | Globulin-1 S allele precursor [*Zea mays*] | gi\|195658 | 81 | 5 | 2/1 | 49.93 | 6.16 | **0.03** | 0.23 |
| 4003 | 06.20 | Globulin-1 S allele precursor [*Zea mays*] | gi\|195658011 | 99 | 10 | 4/1 | 49.89 | 6.16 | **0.06** | **0.14** |

**Table 3** continued

| Spot No | Function category | Protein name | Accession No | Mascot score | % Cover | Peptides +/− | MW [kDa] | p*I* | Fold change $p \leq 0.01$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | B/C | B/D |
| 5304 | 06.13 | Proteasome subunit alpha type [*Zea mays*] | gi\|414865138 | 330 | 39 | 13/9 | 27.4 | 6.1 | **0.04** | |
| 4203 | 06.13 | Unnamed protein product [*Zea mays*] 97 % identities to Proteasome subunit beta type 6 precursor [*Zea mays*] | gi\|229612088 | 169 | 22 | 6/3 | 26.16 | 5.47 | **0.15** | 0.29 |
| *Transporters* | | | | | | | | | | |
| 1702 | 07.25 | Hypothetical protein ZEAMMB73_715164 [*Zea mays*] 96 % identities to 26S protease regulatory subunit 6A homolog [*Oryza sativa* Japonica Group] | gi\|413944465 | 77 | 13 | 6/2 | 47.78 | 4.94 | **0.10** | **0.11** |
| *Disease/defence* | | | | | | | | | | |
| 1001 | 11.06 | Glyoxalase family protein superfamily [*Zea mays*] | gi\|195604212 | 314 | 42 | 8/5 | 15.07 | 5.47 | **0.03** | **0.03** |
| 3401 | 11.06 | Glyoxalase family protein isoform 2 [*Zea mays*] | gi\|413917004 | 579 | 55 | 19,13 | 32.32 | 5.82 | **0.08** | |
| 4502 | 11.05 | General stress protein 39 SDR family protein [*Zea mays*] | gi\|195659117 | 709 | 47 | 29/17 | 32.92 | 5.78 | **0.15** | 0.35 |
| 4601 | 11.02 | Protein Z [*Zea mays*] | gi\|195606550 | 141 | 16 | 10/5 | 42.16 | 5.52 | **0.07** | 0.33 |
| 5102 | 11.05 | 22.0 kDa class IV heat shock protein precursor [*Zea mays*] | gi\|195644560 | 77 | 37 | 7/1 | 22.88 | 6.01 | **0.10** | **0.11** |
| 5201 | 11.06 | Glutathione transferase III(a) [*Zea mays*] | gi\|4468792 | 107 | 27 | 4/2 | 23.79 | 9.33 | **0.04** | **0.20** |
| 6702 | 11.05 | Formate dehydrogenase 1 [*Zea mays*] | gi\|413953926 | 95 | 14 | 4/2 | 41.39 | 6.32 | **0.04** | **0.01** |
| 7501 | 11.05 | Hypothetical protein ZEAMMB73_578099 [*Zea mays*] 78 % identities to Stress responsive protein [*Zea mays*] | gi\|414866592 | 268 | 22 | 15/10 | 38.37 | 6.3 | **0.13** | **0.15** |
| *Secondary metabolism* | | | | | | | | | | |
| 1303 | 20.1 | Caffeoyl-CoA O-methyltransferase 1 [*Zea mays*] | gi\|413925228 | 109 | 19 | 3/0 | 27.3 | 5.09 | **0.13** | |
| *Unclassified* | | | | | | | | | | |
| 4403 | 13 | Secreted protein [*Zea mays*] | gi\|413951152 | 238 | 44 | 12/7 | 27.33 | 5.84 | **0.06** | **0.06** |

The table contains: spots excised from gels and numbered by PDQuest Software used for quantitative gel analysis; function category consistent with *Nature* (Bevan et al. 1998); protein names and accession numbers consistent with NCBI database; Mascot score, sequence coverage (%), number of matched/unmatched (+/−) peptides, MW [kDa] and p*I* values; and relative abundance fold change in comparison with hydroprimed (B) seeds ($p \leq 0.01$)—bolded number indicate ≥5-fold changes in protein expression in the compared variants

seedlings, especially if starch is seed storage material, as is in the case of maize.

The appearance of new isomerases (1801), cupins (8103) and chaperonins (2802) involved in efficient protein destination and storage was also observed (Table 1; Fig. 1BCD). Cupins belong to superfamily of functionally diverse proteins, including seed storage proteins, auxin binding proteins, enzymes, transcription factors, and stress-related proteins, which are involved in seed germination and seedlings development (Dunwell et al. 2004; Gong et al. 2013). Fu et al. (2011) suggested that cupin proteins, such as native germin and germin-like proteins, protected plant cells from the oxidative stress during seed germination.

Similar to the observations by other authors (Gallardo et al. 2001; Gong et al. 2013) in the conditioned plant material, a significant synthesis of so-called stress proteins (5 new spots) was indicated. They belonged to short-chain dehydrogenases/reductases family (SDR; general stress proteins 39—3502, 4502), heat shock proteins (HSP 70 kDa—3901), thioredoxins (TRX; thioredoxin homolog 2—6001) and anaerobic proteins (ANPs; alcohol dehydrogenase 1 isoform XI—6902) (Table 1, Fig. 1BCD).

HSPs were first described as associated with heat shock, but now, it is known that they are also overexpressed during other stresses, i.a. exposure to cold, UV light, and during wound healing or tissue remodelling. They may participate in intracellular distribution and degradation of

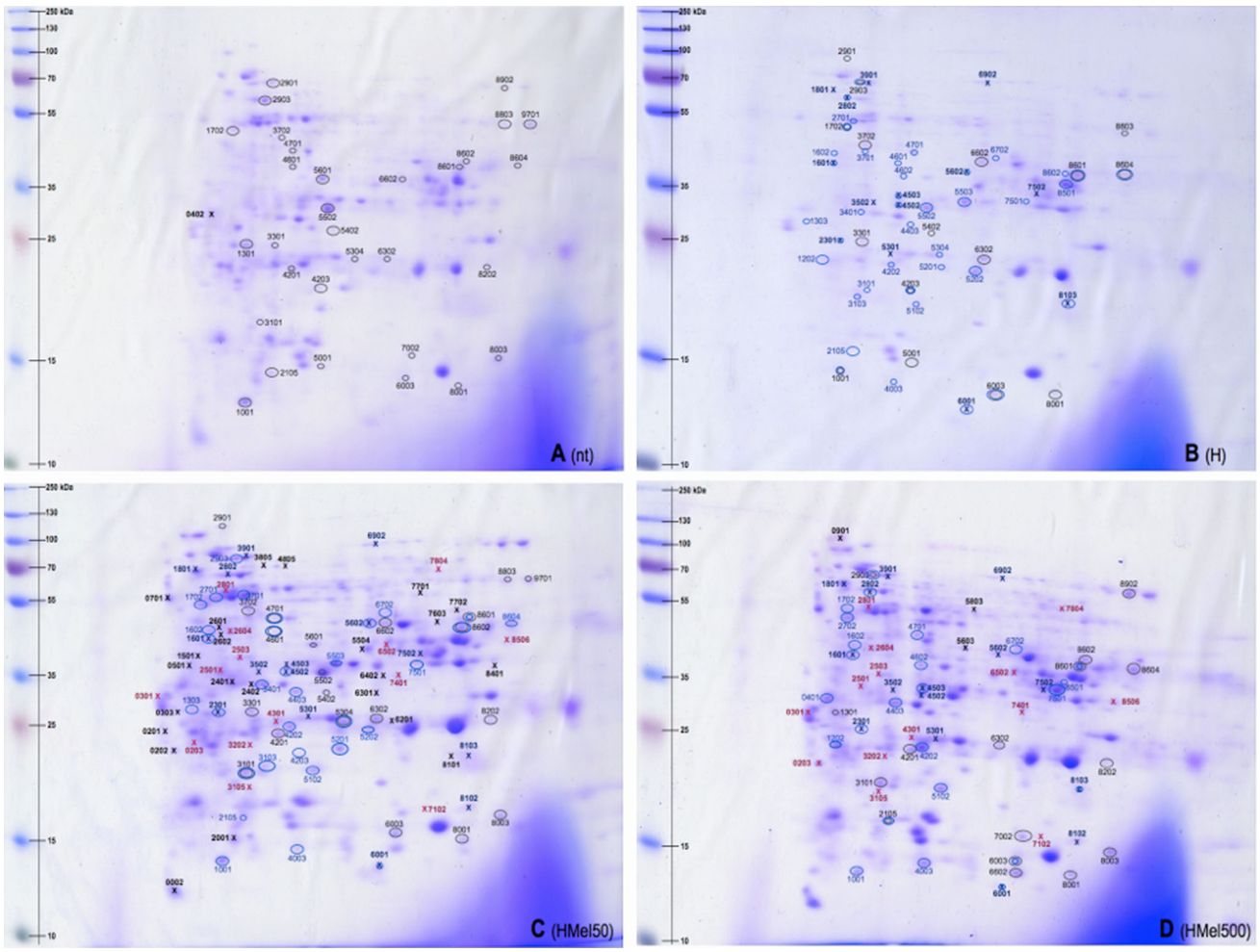



**Fig. 1** Analytical gels of IEF/SDS–PAGE (2D) separation of proteins from: control nt (**A**), hydroprimed (**B**), hydroprimed with melatonin 50 µM (**C**) and 500 µM (**D**) seeds germinated in darkness at 25 °C for 24 h. The proteins were separated by first dimensional pH 3–10 non-linear IPG strips and 12 % vertical slab gels in the second dimension (ImperialTM Protein Stain). The proteins were numbered (PDQuest Software, BioRad) in a preparative 2D electrophoresis gel and excised for MS/MS analysis, corresponding to the spot/proteins in Tables 1, 2 and 3. According to the qualitative analysis, the *black X symbols* mean proteins characteristic only of particular gels (A or B or C or D), *blue X symbols* mean proteins characteristic of all hydroprimed seeds (BCD), and *red X symbols* means proteins characteristic of the seeds hydroprimed with melatonin (CD) (see Table 1). According to the quantitative analysis, ellipses are used to mark proteins five times upregulated or downregulated: *black* for A to B, C, D (see Table 2), *blue* for B to C, D (see Table 3)

proteins, thus perform chaperone activity (Timperio et al. 2008), but also act as molecular factors in signal transduction cascades, affording a protective function during seed germination (Wang et al. 2004).

TRX is a small conservative protein, which causes the changes in redox state of target proteins by reversible oxidation of di-thiol—an active site exposed at the protein's surface (at least 30 target proteins functioning are regulated this way). Some of TRXs are transcription factors and enzymes involved in plant oxidative stress response. They can also limit stress via direct hydrogen peroxide and certain radicals scavenging, and/or by acting as a reductant for peroxiredoxins (Vieira Dos Santos and Rey 2006).

ANPs mostly represent enzymes of the glycolytic and alcohol fermentation pathways. Their upregulation occurs in response to hypoxia/anoxia stress, but cross talk with other kinds of stresses is possible too (Grover et al. 2001).

Interestingly, under the influence of seed hydroconditioning aspartic proteinase oryzasin-1 precursor (0402) disappeared from embryo. It was characteristic only of the axes from control nt seeds (Table 1; Fig. 1A). This plant phytepsin is a homolog of lysosomal pepsins of mammals, and it appears in seeds but also in leaves, stems, flowers and roots. Phytepsin could be overexpressed in plant tissues undergoing apoptosis. It may influence metabolic turnover and other protein processing events.

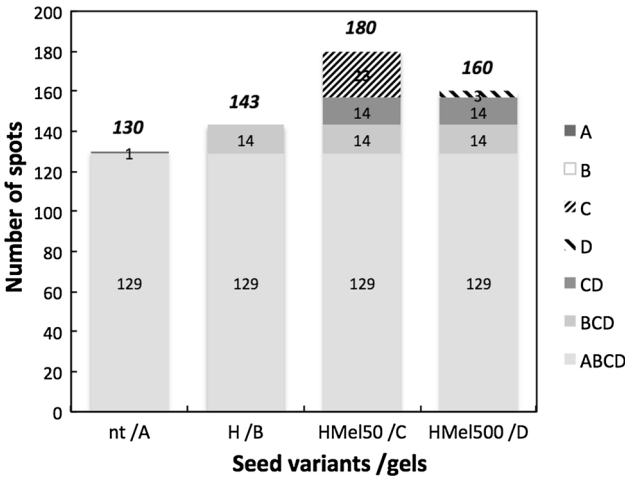



**Fig. 2** Number of protein spots in particular gels: *A*, *B*, *C*, *D*, corresponding with seed variants: nt, H, HMel50, HMel500, respectively. The numbers of protein spots which occurred in certain types of gels were highlighted in different pattern: (1) common for all seeds (*ABCD*), (2) common for the hydroprimed seeds (*BCD*), (3) common for the melatonin treated seeds (*CD*) and (4) unique for the particular seed variants (*A* or *B* or *C* or *D*). *Bolded italics numbers* above the *bars* indicate the total number of protein spots in the particular gels

There were no more proteins characteristic only of the control seed axes (gel A); nevertheless, several enzymes of primary metabolism showed here overexpression comparing with the axes of conditioned seeds (gels B, C, D), i.e. adenylate cyclase—1301 and cytoplasmic maleate dehydrogenase (MDH)—5601 (Table 2; Fig. 1A). Adenylate cyclase (AC) is an enzyme that plays a crucial regulatory function in almost all cells. It facilitates the conversion of ATP to 3′ 5′- cyclic AMP (cAMP) and pyrophosphate. The resultant cAMP operates as a regulatory factor by activation of cAMP-binding proteins, transcription factors, or other enzymes (e.g. cAMP-dependent kinases). MDH acts in the Krebs cycle. It catalyses: the conversion of malate to oxaloacetate and also replenishing level of oxaloacetate via pyruvate reductive carboxylation.

An embryonic DC-8-like protein—8803 also showed significant overexpression (Table 2; Fig. 1A); generally, its content decreases during the initial phase of maize seeds imbibition (Tnani et al. 2012), and this phase, in a case of hydroconditioned seeds, took place just during priming hydration.

It should also be noted that three of the stress-related proteins that occurred in embryos of all seed variants showed overexpression in the control ones: general stress protein 39—5502; heat shock 70-kDa mitochondrial-like protein—2901; glyoxalase family protein—1001; moreover, another protein of unknown function—secreted protein—5402 also showed significant overexpression (Table 2; Fig. 1A). However, the above-described metabolism improvement in nt seed axes is not so significant compared with the effects of the aforementioned completely new stress proteins in primed seed axes (Table 1; Fig. 1BCD) as well as to a significant overexpression in the primed seed embryos of four other proteins that were present in all seed variants: protein Z—4601; 16.9-kDa class I heat shock protein 1—5001; stress-inducible membrane pore protein—7002; and other (different pI values in comparison with spot 5502) general stress protein 39—3301 (Table 2; Fig. 1).

Moreover, the enzymes most important for the energy metabolism, i.e.: triose-phosphate isomerase—4201; cytosolic 3-phosphoglycerate kinase—4701; fructose-bisphosphate aldolase—8601; fructose-bisphosphate aldolase cytoplasmic isozyme—8604; succinyl-CoA ligase alpha-chain 2—8602, occurred in much greater quantities in the embryos of conditioned seeds (Table 2, Fig. 1BCD). Triosephosphate isomerase (TPI) plays a significant role in glycolysis and is fundamental for efficacious energy generation. It makes possible interconversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. Phosphoglycerate kinase (PGK) enables the phosphate group transfer from 1,3-bisphosphoglycerate into ADP, forming ATP and 3-phosphoglycerate. This is one of the two substrate-phosphorylation reactions during glycolysis. Moreover, fructose-1,6-bisphosphate aldolase catalyses the cleavage of fructose 1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP). The last of mentioned above enzymes—succinyl-CoA ligase [GDP-forming]—participates in the Krebs cycle and propanoate metabolism, and thus, it also affects the improvement of energy metabolism.

In addition, some kinases crucial for signals transduction were also overexpressed in axes of primed seeds, e.g.: nucleoside diphosphate kinase—6003 and nucleoside diphosphate kinase 4—8001; (Table 2, Fig. 1BCD). Nucleoside diphosphate kinases (NPcs) are involved in the nucleoside triphosphates (NTPs) generation, and they act in various regulatory processes related to proliferation, differentiation and development. For example, they are essential for DNA and RNA synthesis—thus crucial for metabolism of macromolecules, cell division, and growth.

As it was mentioned earlier, it is difficult to compare proteomes of *Arabidopsis* seeds with those of monocotyledonous corn, especially that in research concerning *Arabidopsis,* whole seeds were usually used, while in the case of corn seeds, we were able to isolate from them the parts which were most active during germination, i.e. embryonic axes. The one important effects of hydropriming common for both plants considered in overexpression of glyceraldehyde-3-phosphate-dehydrogenase in *Arabidopsis* and appearance of its additional isoforms in corn axes (Table 1; Gallardo et al. 2001). In *Arabidopsis,* this enzyme was correlated with the secondary desiccation

(drying after seed priming hydration/imbibition) (Gallardo et al. 2001). Moreover, it was also reported to be a molecular seed vigour marker in *Beta vulgaris* seeds (Catusse et al. 2011).

In our hydroprimed plant material WD-40 proteins, aconitase and such a great amount of seed storage proteins subunits (12S) as in *Arabidopsis* (Gallardo et al. 2001) were not observed. The latter ones might not be detected because of storage tissues absence in our material (only axes). However, as concerns storage proteins, we observed that cupin translocation to the axes was much more efficient in the primed corn (especially when priming was supplemented with melatonin) than in the control.

Moreover, overexpression of tubulin and catalase (CAT) observed in *Arabidopsis* was not noted in our material. Out of energetic metabolism enzymes, other than phosphoenolopyruvate carboxykinase were overexpressed in corn. On the other hand, increase in HSP proteins was observed both in *Arabidopsis* and corn; however, in corn, not only 18 kDa but also 22- and 70-kDa HSPs were detected. (Tables 1, 2; Gallardo et al. 2001). In general, the spectrum of anti-stress and defence proteins induced by priming was wider in corn than in *Arabidopsis.*

It is difficult to find precise similarities between seed plant standard (*Arabidopsis*) and our plant material in terms of their reactions to hydropriming, but it is certainly possible to compare changes in the proteins of specific functional groups, such as: protein mobilising reserves (different enzymes depending on the seed storage material), those responsible for energy processes in cells (glycolytic enzymes, and involved in the Krebs cycle) as well as anti-stress and defence ones (e.g. various HSP, LEA, TRX, and ANP).

To sum up, significant improvement of metabolic processes involved in energy production as well as biosynthesis of anti-stress/stress-induced protective proteins before the occurrence of stress conditions are the facts that explain positive effects of hydroconditioning on seed quality.

### Influence of melatonin on axes proteome in germinated maize grains

Presented results are the first that describe influences of exogenous melatonin on embryo's proteome during early stages of seed germination. Only one paper concerning proteomic modifications during leaf senescence in *Malus hupehensis* treated with exogenous melatonin has been published so far (Wang et al. 2014). Thus, those researches have been performed with completely different plant materials and focused on different developmental stages and processes (senescence).

Application of melatonin during the hydropriming treatment resulted in stronger improvement of energy metabolism in axes—compared with the only hydroprimed seed variant. Especially, the dose of 50-μM melatonin provoked a synthesis of additional six isoforms of enzymes involved in the sugar metabolism through glycolysis and gluconeogenesis processes (i.e. fructokinase 1—1501; 2,3-bisphosphoglycerate-independent phosphoglycerate mutase isoform X1—4805; triosephosphate isomerase, cytosolic—3805; aldolase1 = fructose-bisphosphate aldolase cytoplasmic isozyme—7701; glyceraldehyde-3-phosphate dehydrogenase1 isoform 1—7603 and glyceraldehyde-3-phosphate dehydrogenase1 cytosolic isoform X1—7702) and also two of the crucial enzymes in the citric acid cycle (succinyl-CoA ligase beta-chain—2602 and malate dehydrogenase—5504) (Table 1; Fig. 1C).

A very interesting result, according to Szafranska et al. (2012) research, is the fact confirming that melatonin applied to seeds stimulates polyphenol metabolism. Only in the embryos, proteome of melatonin treated seeds synthesis of 2-dehydro-3-deoxyphosphooctonate aldolase (8506) was observed (Table 1; Fig. 1CD). This enzyme is involved in the shikimate pathway. Members of its family catalyse first steps during biosynthesis of aromatic amino acids. Secondary metabolites accumulation often occurs in plants subjected to different stresses, various elicitors, and also by direct signal molecules action. It is widely known that secondary metabolites are essential for plant interaction with its environment, for adaptation and defence against stress conditions (Bennett and Wallsgrove 1994; Janas et al. 2009b; Ramakrishna and Ravishankar 2011).

In the case of embryos from seeds pretreated with melatonin, a significant improvement of the processes related to the biosynthesis, modifications, and protein destination and storage was observed. In axes of both seed variants treated with melatonin, additional transcription factor 60S acidic ribosomal eukaryotic protein P0 (2503), which composed large subunit of ribosome (it forms main structure in the large subunit, and it is believed to be linked with GTPase activities during protein biosynthesis), as well as directly involved in translations elongation factor 1-delta 1 (EF1B'—0301), occurred. Additionally, elongation factor 1-beta (EF1B—0303) was characteristic for axes from HMel50 seeds (Table 1, Fig. 1CD). Both EF1B' and EF1B catalyse the exchange of GDP bound to the G-protein, for GTP, and this is vital step in elongation processes during protein formation. EF1A—the overexpression of which was observed in all variants of conditioned seed axes (Table 2)—is related to deliver the aminoacyl-tRNA to the ribosome. Eukaryotes eEF1-alpha interacts with the cytoskeleton by actin and may thereby play important role in cellular transformation or apoptosis. This with the simultaneous occurrence of proliferating-associated protein

2G4 (7804), actin-depolymerizing factor 3 (2001), GTPase-activating protein AGD11 (8101) and of DNA repair protein RAD 23 (0701) in HMel50 seeds (Table 1; Fig. 1C) resulted in intense proliferation and better cell growth of the embryos pretreated with melatonin.

As mentioned earlier, already seed hydroconditioning treatment stimulated the biosynthesis of stress proteins in embryos (5 additional spots in B, C, D gels), while melatonin added at the same time provoked additional 3 protein spots in both melatonin seed variants (small HSP—3105, 7102 and thioredoxin TRp26 isoform 4—0203) (Table 1, Fig. 1CD) and 7 anti-stress proteins in HMel50 seeds (co-chaperone protein SBA1—0201; translationally controlled tumour protein (TCTP) homolog—0202; universal stress family protein (USP)—0501; hypothetical protein ZEAMMB73_682876—2601; 1-Cys peroxiredoxin antioxidant PER1—6201; glyoxalase I—2402; and esterase D—6402) (Table 1; Fig. 1C).

SBA1 is an alpha-crystallin-HSPs p23-like protein. TCTP is a highly conserved protein associated with a variety of cellular processes. Primarily, it acts as a molecule that prevents cell death via calcium binding and microtubule stabilization. TCTP also reduces cellular stress acting as molecular chaperone like HSP.

USP family is composed of small cytoplasmic proteins highly expressed as response to various stress factors. USP improves cell survival during prolonged exposure to harmful conditions, and may provide an overall "stress endurance". Hypothetical protein ZEAMMB73_682876 showed 88 % identities to protein DJ-1 B-like homolog [*Setaria italica*]. This DJ-1 homolog exhibits chaperone-like properties, and it is activated together with genes related to oxidative stress response in plants.

In addition, peroxiredoxins (PRXs) are involved in redox status regulation in cells. Subfamily of 1-Cys-PRX is composed of PRXs containing only one conserved cysteine. This amino acid operates as peroxidatic agent. Thus, PRXs are homodimeric thiol-specific antioxidants, which play defensive role by reduction of organic hydroperoxides, peroxynitrite, and excess of hydrogen peroxide in plant cells. While glyoxalases and esterases are typical detoxifying enzymes; glyoxalase I (other name: lactoylglutathione lyase) catalyses the hemithioacetal isomerization, formed by a glutathione and 2-oxoaldehyde, to S-D-lactoyl-glutathione. It is considered as a part of two-step system (called glyoxalase system) for detoxication of methylglyoxal (toxic side product of glycolysis).

Esterases and lipases interact with carboxylic esters. Many of them participate in the first phase of xenobiotics metabolism (especially toxins). The resulting this way carboxylates is then joined by other enzymes to increase their solubility and/or eventually for their excretion.

Quantitative analyses revealed that although the two glycolytic enzymes were overexpressed in axes of hydroprimed seeds (H) (cytosolic glycerqaldehyde-3-phosphate dehydrogenase—8501 and fructose-bisphosphate aldolase, cytoplasmic isozyme—8604) (Table 3; Fig. 1), their other isomeric forms were present in much greater quantity in axes of seeds conditioned with melatonin (glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic—5602 and fructose-bisphosphate aldolase—8601) (Table 3; Fig. 1). In general, comparing the enzymes involved in energy metabolism a significant number (6) was overexpressed in the seeds hydroconditioned with melatonin compared with those hydroprimed without it. They were 6-phosphogluconolactonase isoform 2—2301, enolase—2701, triosephosphate isomerase, cytosolic—4202, malate dehydrogenase—4602, cytosolic 3-phosphoglycerate kinase—4701 and succinyl-CoA ligase alpha-chain 2—8602 (Table 3; Fig. 1).

Many proteins involved in protein destination, storage and turnover also were overexpressed under the influence of melatonin compared all hydroprimed seeds (i.e. chaperonin 60—2802, mitochondrial chaperonin-60—2903, globulin 2—3101, globulin-1 S allele precursors—3101 and 4003, proteasome subunit alpha type—5304, and unnamed protein product similar to proteasome subunit beta type 6 precursor—4203) (Table 3; Fig. 1CD). In the axes of hydroconditioned seeds in this functional protein group, only one cupin (8103) was overexpressed.

As concerns stress-related and defence proteins, 4 of them (i.e. HSP 26—1202, 1-Cys peroxiredoxin antioxidant—5202, general stress protein 39—5502, thioredoxin h2 protein—6001) were overexpressed in the hydroprimed seed axes (Table 3; Fig. 1B) and 8 in the axes from seeds hydroprimed with melatonin (i.e. glyoxalase family protein superfamily—1001, glyoxalase family protein isoform 2—3401, other general stress protein 39—4502, protein Z—4601, 22.0 kDa class IV HSP precursor—5102, glutathione transferase III(a)—5201, formate dehydrogenase 1—6702, and hypothetical protein ZEAMMB73_578099 similar to stress responsive protein—7501) (Table 3; Fig. 1CD).

Again, it was confirmed that melatonin applied to the seed affects polyphenols metabolism. In axes of HMel50 seeds, significant overexpression of caffeoyl-CoA *O*-methyltransferase 1 (1303) was observed in comparison with H seeds (Table 3; Fig. 1BC). It is an enzyme that catalyses the reaction between *S*-adenosyl-L-methionine and caffeoyl-CoA, which are transformed to *S*-adenosyl-L-homocysteine and feruloyl-CoA. A large number of secondary metabolites are generated via a step involving this enzyme. In general, it participates in phenylpropanoid biosynthesis.

## Conclusions

To sum up (Fig. 2), hydropriming (H, HMel50, HMel500) provoked biosynthesis of 14 new, characteristic proteins in embryonic axes of the grains germinated at optimal temperature compared with the non-treated ones. When this pre-sowing treatment was supplemented with melatonin (HMel50, HMel500), additional 14 proteins occurred. Moreover, in the seed variant HMel50 (optimal melatonin concentration)—23 and in HMel500—3 characteristic spots were noticed. The majority of additional proteins belonged to energy metabolism enzymes, proteins involved in proteome plasticity via improving protein synthesis, folding, destination and storage, and—most importantly—defence, anti-stresses and detoxifying proteins.

Proteins belonging to these functional groups are frequently ranked as seed quality markers. Particularly, important are those considered to be anti-stress proteins, e.g. HSP, LEA, TRX, and other general stress proteins (Gallardo et al. 2001; Catusse et al. 2011; Wu et al. 2011). Especially, many of these proteins were determined in the corn seeds conditioned with melatonin.

In the literature, the beneficial effect of antioxidant enzymes, i.a. superoxide dismutase (SOD), CAT, glutathione-*S*-transferase (GST) on seed quality improvement, was underlined (Gallardo et al. 2001; Catusse et al. 2011). However, in our study, we did not observe any differences in the synthesis of these enzymes in the compared seed variants. It seems possible that supplementation of the seeds with melatonin—a highly effective, small molecule antioxidant—and overexpression of other antioxidant and detoxifying factors, such as TRXs, glioxylases, and improved polyphenol biosynthesis, were enough to regulate internal redox balance, and additional antioxidant enzymes were not needed in the conditioned (improved) seeds.

Moreover, the following factors are important for better seed vigour and quality, on the one hand, efficacious reserve mobilisation, and thus increase in the seed storage proteins, such as cupins and/or globulins—their translocation into embryonic axes of the conditioned corn seeds was noted in the presented study—and, on the other hand, the highly productive energy metabolism. With regard to that overexpression of gliceraldehyd-3-phosphate-dechydrogenase observed by us was also mentioned by other authors (Gallardo et al. 2001; Catusse et al. 2011; Wu et al. 2011), however, other glycolytic or Krebs cycle enzymes indicated as vigour markers were different depending on the seed type. In addition, Catusse et al. (2011) also pointed to the voltage-dependent anion channels (VDACs) as these markers, which were confirmed neither by other authors nor by us.

It seems that when determining markers of seed quality attention should also be paid to the proteins involved in sulphur assimilation and metabolism. Similarly, with regard to that also various proteins were indicated by scientists, i.a. phosphoserine aminotranspherase, *S*-adenosyl-L-methionine synthetase, serine hydroxymethyl transpherase (Catusse et al. 2011), but in most reports as well as in our work, cysteine synthase appeared (Gallardo et al. 2001; Catusse et al. 2011; Wu et al. 2011).

Unfortunately, it seems that the determination of specific seed quality markers will be really difficult due to the variety of seed material subjected to tests under different conditions. However, it is possible to indicate functional groups in which overexpression of different proteins could be beneficial for seed vigour improvement.

Indicating such protein groups and also specific examples for the tested plant material (Table 1), we partially explained why the hydroprimed corn seeds—especially hydroprimed with melatonin, and the seedlings grown from them were stronger in comparison with the non-treated ones, and so quickly and efficiently adapted to the changing environmental conditions (Posmyk et al. 2008, 2009a, b; Janas et al. 2009a). They could be a priori prepared to defence against potential harmful condition. In embryonic axes during the initial state of growth, even under optimal conditions, a number of antioxidative, detoxifying, and chaperon proteins were synthesized. Moreover, the supply of energy from seed storage substances was pretty intensified. The presented results for the first time direct explain how melatonin acts in seed axes as plant stress defence factor. It could be taken as a part of explanation why various plant species with higher content of melatonin have shown improved tolerance to stress (Park et al. 2013; Bajwa et al. 2014; Zhang et al. 2015).

Since melatonin is safe for environment (non-toxic, of natural origin, biodegradable) as well as being inexpensive, it is using as a supplement of various priming methods may be a reliable and cost-effective manner for beneficial seed quality modifications and could be economically favourable for organic farming (Janas and Posmyk 2013). Plant biostimulation by melatonin application is a promising tool to enhance ecological crops and to support safety-food production.

**Author contributions statement** Izabela Kołodziejczyk—all experiments concerning corn seeds, protein extraction, purification and 2D separation, data acquisition and PDQuest analysis/interpretation. Katarzyna Dzitko—methodological consultant and technical support, optimisation of 2D separation method for corn seed proteins. Rafał Szefczyk—LC–MS/MS and statistical analysis, database searches. Małgorzata M. Posmyk—work con-

ception, obtaining of funding, data analysis and interpretation, drafting of the manuscript, responsible for the integrity of the work.

**Acknowledgments** Presented research was supported by the National Science Centre of Poland as NCN NN310 111940 project.



## References

Arnao MB, Hernández-Ruiz J (2007) Melatonin promotes adventitious and lateral root regeneration in etiolated hypocotyls of *Lupinus albus* L. J Pineal Res 42:147–152

Bajwa VS, Shukla MR, Sherif SM, Murch SJ, Saxena PK (2014) Role of melatonin in alleviating cold stress in *Arabidopsis thaliana*. J Pineal Res 56:238–245

Basak A (2008) Biostimulators—definitions, classification and legislation. In: Gawronska H (ed) Biostimulators in modern agriculture. Editorial House Wies Jutra, Warszawa, pp 7–17

Bennett RN, Wallsgrove RM (1994) Secondary metabolites in plant defence mechanisms. New Phytol 127:617–633

Bevan M, Bancroft I, Bent E et al (1998) Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. Nature 391:485–488

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dry binding. Anal Biochem 72:248–254

Bradford KJ, Bewley JD (2002) Seeds: Biology, technology and role in agriculture. In: Chrispeels MJ, Sadava DE (eds) Plants, genes and crop biotechnology. Jones and Bartlett, Boston, pp 210–239

Catusse J, Meinhard J, Job C, Strub JM, Fischer U, Pestsova E, Westhoff P, Van Dorsselaer A, Job D (2011) Proteomics reveals potential biomarkers of seed vigor in sugarbeetrt. Proteomics 11:1569–1580

Chen K, Arora R (2013) Priming memory invokes seed stress-tolerance. Environ Exp Bot 94:33–45

Chen Q, Qi WB, Reiter RJ, Wei W, Wang BM (2009) Exogenously applied melatonin stimulates root growth and raises endogenous indoleacetic acid in roots of etiolated seedlings of *Brassica juncea*. J Plant Physiol 166:324–328

Di Girolamo G, Barbanti L (2012) Treatment conditions and biochemical processes influencing seed priming effectiveness. Ital J Agron 7:178–188

Dunwell JM, Purvis A, Khuri S (2004) Cupins: the most functionally diverse protein superfamily? Phytochemistry 65:7–17

Eskandari H (2013) Effects of priming technique on seed germination properties, emergence and field performance of crops—a review. Int J Agron Plant Prod 4:454–458

Fu Z, Jin X, Ding D, Li Y, Fu Z, Tang J (2011) Proteomic analysis of heterosis during maize seed germination. Proteomics 11:1462–1472

Gallardo K, Job C, Groot SP, Puype M, Demol H, Vandekerckhove J, Job D (2001) Proteomic analysis of *Arabidopsis* seed germination and priming. Plant Physiol 126:835–848

Gong F, Wu X, Wang W (2013) Comparative proteomic identification of embryo proteins associated with hydropriming induced rapid-germination of maize seeds. Plant Omics 6:333–339

Grover A, Kapoor A, Lakshmi OS, Agarwal S, Sahi C, Katiyar-Agarwal S, Agarwal M, Dubey H (2001) Understanding molecular alphabets of the plant abiotic stress responses. Curr Sci 80:206–216

Hernández-Ruiz J, Arnao MB (2008) Melatonin stimulates the expansion of etiolated lupin cotyledons. Plant Growth Regul 55:29–34

Hernández-Ruiz J, Cano A, Arnao MB (2005) Melatonin acts as a growth-stimulating compound in some monocot species. J Pineal Res 39:137–142

Janas KM, Posmyk MM (2013) Melatonin, an underestimated natural substance with great potential for agricultural application. Acta Physiologiae Plantarum 35:3285–3292

Janas KM, Ciupińska E, Posmyk MM (2009a) Melatonin applied by hydropriming, as phytobiostimulator improving corn (*Zea mays* L.) seedlings growth at abiotic stresses conditions. In: Li S, Wang Y, Cao F, Huang P, Zhang Y (eds) Progress in environmental science and technology, vol II A. Science Press USA Inc., pp 383–388, ISBN 978-7-03-024459-8

Janas KM, Amarowicz R, Zielińska-Tomaszewska J, Kosinska A, Posmyk MM (2009b) Induction of phenolic compounds in two lentil cultivars with different tolerances to copper ions. Acta Physiol Plant 31:587–595

Jisha KC, Vijayakumari K, Puthur JT (2013) Seed priming for abiotic stress tolerance: an overview. Acta Physiol Plant 35:1381–1396

Kołodziejczyk I, Dzitko K, Szewczyk R, Posmyk MM (2016) Exogenous melatonin improves corn (*Zea mays* L.) embryo proteome in seeds subjected to chilling stress. J Plant Physiol 193:47–56

Mabhaudhi T, Modi AT (2011) Can hydropriming improve germination speed, vigour and emergence of maize landraces under water stress? J Agric Sci Technol B 1:20–28

Park S, Lee DE, Jang H, Byeon Y, Kim YS, Back K (2013) Melatonin rich transgenic rice plants exhibit resistance to herbicide-induced oxidative stress. J Pineal Res 54:258–263

Posmyk MM, Kuran H, Marciniak K, Janas KM (2008) Pre-sowing seed treatment with melatonin protects red cabbage seedlings against toxic copper ion concentrations. J Pineal Res 45:24–31

Posmyk MM, Bałabusta M, Janas KM (2009a) Melatonin applied by osmopriming, as phytobiostimulator improving cucumber (*Cucumis sativus* L.) seedlings growth at abiotic stresses conditions. In: Li S, Wang Y, Cao F, Huang P, Zhang Y (eds) Progress in environmental science and technology, vol II A. Science Press USA Inc., pp 362–369, ISBN 978-7-03-024459-8

Posmyk MM, Bałabusta M, Wieczorek M, Sliwinska E, Janas KM (2009b) Melatonin applied to cucumber (*Cucumis sativus* L.) seeds improves germination during chilling stress. J Pineal Res 46:214–223

Rahimi A (2013) Seed priming improves the germination performance of cumin (*Cuminum syminum* L.) under temperature and water stress. Ind Crops Prod 42:454–460

Ramakrishna A, Ravishankar GA (2011) Influence of abiotic stress signals on secondary metabolites in plants. Plant Signal Behav 6:1720–1731

Sarrou E, Therios I, Dimassi-Theriou K (2014) Melatonin and other factors that promote rooting and sprouting of shoot cuttings in *Punica granatum* cv. Wonderful. Turk J Bot 38:293–301

Szafrańska K, Glińska S, Janas KM (2012) Changes in the nature of phenolic deposits after re-warming as a result of melatonin pre-sowing treatment of *Vigna radiata* seeds. J Plant Physiol 169:34–40

Timperio AM, Egidi MG, Zolla L (2008) Proteomics applied on plant abiotic stresses: role of heat shock proteins (HSP). J Proteomics 71:391–411

Tnani H, López I, Jouenne T, Vicient CM (2012) Quantitative subproteomic analysis of germinating related changes in the scutellum oil bodies of *Zea mays*. Plant Sci 191:1–7

Vieira Dos Santos C, Rey P (2006) Plant thioredoxins are key actors in the oxidative stress response. Trends Plant Sci 11:329–334

Wang WX, Vinocur B, Shoseyov O, Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci 9:1360–1385

Wang P, Sun X, Xie Y, Li M, Chen W, Zhang S, Liang D, Ma F (2014) Melatonin regulates proteomic changes during leaf senescence in *Malus hupehensis*. J Pineal Res 57:291–307

Wu X, Liu H, Wang W, Chen S, Hu X, Li C (2011) Proteomic analysis of seed viability in maize. Acta Physiol Plant 33:181–191

Zhang N, Zhao B, Zhang H-J, Weeda S, Yang Ch, Yang Z-C, Ren S, Guo Y-D (2013) Melatonin promotes water-stress tolerance, lateral root formation, and seed germination in cucumber (*Cucumis sativus* L.). J Pineal Res 54:15–23

Zhang N, Zhang HJ, Zhao B, Sun QQ, Cao YY, Li R, Wu XX, Weeda S, Li L, Ren S, Reiter RJ, Guo YD (2014) The RNA-seq approach to discriminate gene expression profiles in response to melatonin on cucumber lateral root formation. J Pineal Res 56:39–50

Zhang N, Sun Q, Zhang H, Cao Y, Weeda S, Ren S, Guo YD (2015) Roles of melatonin in abiotic stress resistance in plants. J Exp Bot 66:647–656

Oświadczenia współautorów publikacji **Kołodziejczyk I., Dzitko K., Szewczyk R., Posmyk M.M. (2016)** Exogenous melatonin expediently modifies proteome of maize (*Zea mays* L.) embryo during seed germination. *Acta Physiologiae Plantarum* 38: 146 DOI: 10.1007/s11738-016-2166-y **(IF: 1.760$^{2019}$ - 2.078$^{V}$) (70$^{A}$ pkt-y MNSW),** wchodzącej w skład rozprawy doktorskiej:

| Imię i nazwisko | Szacunkowy udział [%] | Opis działań | Podpis |
|---|---|---|---|
| mgr Izabela Kołodziejczyk | 70 | Przygotowanie nasion – wykonanie hydrokondycjonowania (H, HMel50, HMel500). Hodowla materiału roślinnego w ustalonych warunkach eksperymentu. Ekstrakcja, oczyszczanie i zagęszczanie białek z osi zarodkowych. Separacja i wizualizacja białek techniką 2-D. Analizy i typowanie spotów za pomocą oprogramowania PDQuest. Przygotowanie materiału do analiz LC-MS/MS – izolacja i trawienie białek z żeli. Pozyskanie danych z odpowiednich baz – analiza porównawcza przy użyciu narzędzi MASKOT, NCBI i DELTA-BLAST. Opracowanie, analiza i interpretacja wyników. Przygotowanie manuskryptu. | Kołodziejczyk Izabela |
| dr hab. Katarzyna Dzitko, prof. UŁ | 5 | Konsultacja metodyczna dotycząca rozdziałów 2D. | Dzitko |
| dr Rafał Szewczyk | 10 | Wykonanie analiz LC–MS/MS z powierzonego materiału. Instruktaż w pozyskiwaniu danych z MASCOT, NCBI i przy użyciu algorytmu DELTA-BLAST. | Rafał Szewczyk |
| prof. dr hab. Małgorzata M. Posmyk | 15 | Współtwórca koncepcji pracy. Pomoc metodyczna na różnych etapach analiz. Obliczenia statystyczne. Pomoc w interpretacji wyników i redakcji manuskryptu. | Posmyk |
| Dane biblio-metryczne z dn. 19.05.2021 r. wg. *Web.Sci.*: **IF 1.364$^{2016}$, 2.078$^{V}$; 12** cytowań. (**70$^{A}$** pkt-y MEiN) | | | |

*Załącznik 4*

Journal of Plant Physiology 193 (2016) 47–56

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Physiology

# Exogenous melatonin improves corn (*Zea mays* L.) embryo proteome in seeds subjected to chilling stress

Izabela Kołodziejczyk[a], Katarzyna Dzitko[b], Rafał Szewczyk[c], Małgorzata M. Posmyk[a,*]

[a] Department of Ecophysiology and Plant Development, Faculty of Biology and Environmental Protection, University of Lodz, 12/16 Banacha Str., 90-237 Lodz, Poland

[b] Department of Immunology and Infectious Biology, Faculty of Biology and Environmental Protection, University of Lodz, 12/16 Banacha Str., 90-237 Lodz, Poland

[c] Department of Industrial Microbiology and Biotechnology, Faculty of Biology and Environmental Protection, University of Lodz, 12/16 Banacha Str., 90-237 Lodz, Poland

ARTICLE INFO

*Article history:*
Received 21 September 2015
Received in revised form 15 January 2016
Accepted 18 January 2016
Available online 20 February 2016

*Keywords:*
Antioxidant
Chilling stress
Melatonin
Proteome analysis
Seed priming
*Zea mays*

ABSTRACT

Melatonin (MEL; *N*-acetyl-5-methoxytryptamine) plays an important role in plant stress defense. Various plant species rich in this indoleamine have shown a higher capacity for stress tolerance. Moreover, it has great potential for plant biostimulation, is biodegradable and non-toxic for the environment. All this indicates that our concept of seed enrichment with exogenous MEL is justified. This work concerns the effects of corn (*Zea mays* L.) seed pre-sowing treatments supplemented with MEL. Non-treated seeds (nt), and those hydroprimed with water (H) or with MEL solutions 50 and 500 μM (HMel50, HMel500) were compared. Positive effects of seed priming are particularly apparent during germination under suboptimal conditions. The impact of MEL applied by priming on seed protein profiles during imbibition/germination at low temperature has not been investigated to date. In order to identify changes in the corn seed proteome after applying hydropriming techniques, purified protein extracts of chilling stressed seed embryos (14 days, 5 °C) were separated by two-dimensional electrophoresis. Then proteome maps were graphically and statistically compared and selected protein spots were qualitatively analyzed using mass spectrometry techniques and identified. This study aimed to analyze the priming-induced changes in maize embryo proteome and at identifying priming-associated and MEL-associated proteins in maize seeds subjected to chilling. We attempt to explain how MEL expands plant capacity for stress tolerance.



## 1. Introduction

Crop production technologies based only on the improvement of cultivation techniques are beginning to face limitations resulting from an inability to use the biological potential inherent in the cultivar. Climate change is already beginning to impair maize and wheat production. Higher average temperature fluctuations in most major grain-producing countries are beginning to work against the progress made by improving seeds and farming practices, according to the study published in *Science* by Lobell et al. (2011). Thus, there is a constant search for new solutions to ensure the most favorable conditions for plant growth and development, for instance by limiting various biotic and abiotic stresses, and ultimately to increase yield. The most preferable solution seems to be search for and then the use of natural bio-stimulators together with seed priming.

The latest results obtained by our group suggest that MEL has a great potential for plant biostimulation (Janas and Posmyk,

*Abbreviations:* ACN, acetonitrile; AFMK, *N*1-acetyl-*N*2-formyl-5-methoxykynuramine; Btf3, basal transcription factor 3; CHIP, carboxyl terminus of Hsc70-interacting protein; DDT, DL-dithiothreitol; DOXC, deoxycholate sodium salt; DP, declustering potential; eIF5A, eukaryotic translation initiation factor 5A; EMS, enhanced MS scan; EPI, enhanced product ion scan; ER, enhanced resolution scan; GLPs, germin-like proteins; GSH, reduced glutathione; H, hydroprimed seeds; HMel50, HMel500; HSP, heat shock proteins; IDA, information dependent acquisition method; LEA, late embryogeneis abundant; LMW, low molecular weight; MEL, melatonin; MG, methylglyoxal; NAC, nascent polypeptide-associated complex; NDP, nucleoside diphosphate; NDPk, nucleoside diphosphate kinase; NDPk, nucleoside diphosphate kinases; nt, non-treated seeds; NTP, nucleoside triphosphate; PDIa, protein disulfide isomerase; PMSF, phenylmethanesulfonylfluoride; Prx, peroxiredoxin; RING, really interesting new gene; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDR, short-chain dehydrogenases/reductases; SMP, seed maturation proteins; TCA, trichlorocetic acid; TFA, trifluoroacetic acid; TIM, triosephosphate isomerase; TRX, thioredoxin; UPS, proteins degradation system by ubiquitination.

* Corresponding author.
*E-mail addresses:* izka.kolo@gmail.com (I. Kołodziejczyk), dzika@biol.uni.lodz.pl (K. Dzitko), rszewcz@biol.uni.lodz.pl (R. Szewczyk), posmyk@biol.uni.lodz.pl (M.M. Posmyk).

http://dx.doi.org/10.1016/j.jplph.2016.01.012
0176-1617/

2013). It is a natural, biodegradable and non-toxic, highly conserved molecule present in evolutionarily distant organisms including plants. The pre-sowing seed treatment with MEL protected red cabbage seedlings against toxic Cu ion concentrations (Posmyk et al., 2008) and MEL application to cucumber and corn seeds had a beneficial effect on the seedling growth and crop production of plants that germinated from them, especially those subjected to cold (Posmyk et al., 2009a,b) and water stress (Zhang et al., 2013). MEL plays an important role in plant stress defense (Zhang et al., 2015). Recently, a several papers have tried to explain the molecular mechanism of MEL action in this case (Shi and Chan, 2014; Shi et al., 2015). It was shown that, in *Arabidopsis thaliana* leaves, freezing stress increased endogenous MEL content and it was correlated with the induction of At-ZAT6 expression. Then, the activation of AtZAT6 increased the expression of CBF1-3 directly binding to TACAAT elements of CBF1-3 promoters, and up-regulation of CBF1-3 improved freezing stress resistance in *Arabidopsis* (Shi and Chan, 2014).

Another model of MEL-mediated action in *A. thaliana* leaves assumes that, upon abiotic stresses (cold, salt, and drought) and biotic stress (pathogen infection), an increase in MEL levels is followed by an increase in the transcripts of CBF/DREB1s. This overexpression results in higher transcript levels of multiple stress-responsive genes (COR15A, RD22, and KIN1) and enhanced soluble sugar accumulation (sucrose), thus improving resistance to the above-mentioned stresses (Shi et al., 2015). A complete explanation of all molecular pathways that MEL stimulates and through which it acts in plants will not be easy because MEL appears to be involved into a very dense network of connections in different parts of plants.

Different plant species rich in MEL exhibit a higher capacity for stress tolerance (Park et al., 2013; Bajwa et al., 2014). An increase in melatonin content was also detected in sunflower seeds during sprouting (Cho et al., 2008). Since the germ and reproductive tissues are highly vulnerable to oxidative damage, MEL might be an important component of its antioxidant defense system as a free radical scavenger, especially in dormant and relatively dry tissues of seeds, where enzymes are poorly effective and cannot be up-regulated (Manchester et al., 2000).

Since MEL is soluble in both water and lipids, it may be a hydrophilic and hydrophobic antioxidant. This fact, together with MEL's small size make it particularly able to migrate easily between cell compartments in order to protect them against excessive ROS and RNS. Moreover, recent evidence indicates that the primary MEL metabolites, especially AFMK, also have strong antioxidant abilities. It is documented that the free radical scavenging capacity of MEL extends to its secondary, tertiary and quaternary metabolites (Tan et al., 2007). This process is referred to as the free radical scavenging cascade, which makes MEL, even at low concentrations, highly effective at protecting organisms against oxidative stress. It seems that in an evolutionary sense, the strong antioxidant properties of MEL (Terrón et al., 2001) were its primary role in the defense against unfavorable conditions and in plant stress tolerance, but our results presented in this paper indicate other beneficial effects.

In addition to *in vivo* synthesis, plants can also absorb exogenously provided MEL from the environment and accumulate it at high concentrations (Tan et al., 2007). This, and particularly the evidence that MEL induces resistance to stresses in plants, indicates that our concept of seed enrichment with exogenous MEL is justified.

Despite methodological difficulties, interest in plant proteome research is still growing. Elucidation of changes in protein synthesis and expression under the influence of biotic and abiotic factors can provide a clear understanding of the processes and mechanisms of the plant response to stress. This is a particularly useful and important research tool when the purpose of the study is to find ways to improve the vigor of plants under suboptimal environmental conditions. There is no information concerning the impact of MEL applied by priming on seed protein profiles during germination under low temperature conditions.

This study aimed to analyze the priming-induced changes in the maize embryo proteome and to identify priming-associated and MEL-associated proteins in maize seeds subjected to chilling stress during germination. Comparative proteomic analysis was used to identify differentially expressed proteins in the unprimed seeds (nt) and those hydroprimed (H) and hydroprimed with MEL (HMel50 and HMel500).

## 2. Materials and methods

### 2.1. Plant material

Corn seeds (*Zea mays* L. var. Ambrozja) were provided by TORSEED (Torun, Poland). They were stored in the dark, under dry conditions at room temperature, in tightly closed containers before the experiments started.

### 2.2. Hydroconditioning

Hydroconditioning was performed by the soaking corn seeds at 25 °C in darkness for 3 h, using distilled water or melatonin (50 or 500 μM) water solutions. Seed water content was increased from an initial value of 8.8% (±0.2) to 36.8% (±2.3). The quantity of water needed to increase seed humidity was determined experimentally. Corn seed variants: non-treated (nt-gel A), hydroprimed (H-gel B), hydroprimed with melatonin 50, and 500 μM (HMel50-gel C and HMel500-gel D, respectively). The seeds were re-dried for 3 days at room temperature after hydropriming.

### 2.3. Seed germination

Germination of all seed variants were carried out in darkness at 5 °C for 14 days. Then, axes were isolated, placed into the liquid nitrogen and finally lyophilized.

### 2.4. Protein extraction and purification

Protein extraction was carried out using a homogenizer in an ice bath. Lyophilized tissue (200 mg) was treated with 1.5 mL of extraction buffer 50 mM Tris–HCl (pH 7.7) containing: 0.5 mM DOXC; 10 mM DDT; 10 mM EDTA; 1 mM PMSF. The homogenate was centrifuged: 20 000 g/10 min/5 °C. The pellet was discarded. Proteins from the supernatant were precipitated with 10% TCA and 0.07% merkaptoethanol in acetone, in an ice bath for 45 min. The mixture was centrifuged as noted above. The resulting pellet containing precipitated proteins was washed twice with 0.07% mercaptoethanol in acetone. After the second centrifugation: 3000 × g/5 min./5 °C; sediment was vacuum-dried and then diluted in Rabilloud Buffer containing: 8 M Urea; 4% CHAPS and 65 mM DTT. The protein content was determined in 96-microplates using Bradford's (1976) method. The protein solution was stored at −20 °C and then used for 2-D electrophoresis.

### 2.5. 2-D protein separation and visualization

Quantified proteins (300 mg) were applied to an 11 cm IPG strip (pH 3–10, non-linear gradient ReadyStrip) in a Protean IEF cell (Bio-Rad, Hercules, CA) and in-gel rehydrated for 12 h. The proteins were separated according to their isoelectric point in the IEF-SYS (Scie-Plas Ltd., GB). Isoelectric focusing was performed at 18 °C for 72 h at 0–3000 V gradually changing (first 24 h:0–1500 V; second: 1500–3000; third 3000 V). Prior to the second dimension

electrophoresis, the gel strips were equalibred twice: with Eq solution (Eq: 50 mM Tris–HCl, pH 8.5, 6 M urea, 30% glycerol, 10% SDS) containing 1% DTT for 15 min and then with Eq solution containing 2.5% iodoacetamide. Equilibrated strips were applied to SDS-PAGE (12% analytical gel and 4% stacking gel) and then sealed with 0.5% agarose in 0.5 M Tris–HCl bufer (pH 6.8) and 10% SDS. Protein markers (Thermo Scientific Page-Ruler Plus Prestained Protein Ladder No 26619) were loaded beside the strip before sealing. After solidification, electrophoresis was performed in TV400YK Cooled Twin-Plate Maxi-Gel Electrophoresis Unit (Scie-Plas Ltd., GB) with SDS electrophoresis buffer containing: 25 mM Tris base, 192 mM glycine, 0.1% SDS; for 2 h at 100 V and next for 2 h at 200 V, maintaining a constant temperature 25 °C. At least four independent biological replicates for each sample were performed.

Proteins separated in polyacrylamide gels were visualized by Imperial™ Protein Stain (Pierce). The stained gels were digitalized.

### 2.6. Statistical analysis of gels images

Gel images were analyzed with the software PDQuest version 8.0.1 (BioRad). For each sample, quantification was performed with 3–4 analytical gels originating from three independent biological replicas/experiments ($n$ = at least 9–12). The volume of each spot was normalized to a relative volume, and mean values calculated from repeated data were compared. Qualitative and quantitative analysis sets were created.

Proteins exhibiting the highest reproducible alterations (about 5-fold) were subjected to statistical analysis. The Wilcoxon rank-sum test was chosen to evaluate the statistical significance of the differences (at $p \leq 0.01$) between the control (A) and primed (B, C, D) samples (Table 2) as well as between the hydroprimed (B) and hydroprimed with MEL (C, D) samples (Table 3).

Mass spectrometry was used to identify the proteins that were unique for particular seed variants (Table 1 and those whose amount changed significantly (Tables 2 and 3—the cut-off for differentially regulated proteins was a 5-fold change).

### 2.7. In-gel digestion

The spots of interest were cut out from stained gels and subjected to in-gel tryptic digestion (Promega, Madison, WI) described previously (Huynh et al., 2009). Each gel piece containing the protein of interest was decolorized with a destaining solution: 50 mM $NH_4HCO_3$/ACN (50:50, v/v) for 15 min at room temp. The destaining procedure was repeated until the gel was colorless. Then the gel pieces were washed with ACN to dehydrate the band pieces. The supernatant was discarded. For reducing and alkylation gel pieces were washed with 10 mM DTT in 100 mM $NH_4HCO_3$ and incubate at 56 °C for 30 min, then with 50 mM iodoacetamide in 100 mM $NH_4HCO_3$ and incubate at room temperature for 30 min in dark. Then, the pieces were washed twice alternately with 100 mM $NH_4HCO_3$ and ACN. Each time the supernatant was poured. The band pieces were dried at room temperature and rehydrated in 25 mM $NH_4HCO_3$ with trypsin (Promega) at 37 °C overnight. After the digestion gel bands were extracted with 2% ACN/0.1% TFA two times to collect the remaining peptides. The collected supernatant was used for further analysis.

### 2.8. LC–MS/MS analysis

LC–MS/MS analysis was done on Eksigent ExpressHT Micro LC and AB Sciex QTRAP 3200 mass spectrometer with a microspray ESI ion source installed. Tryptic digests were ultrasonicated for 30 sec, shaken and centrifuged for 5 min. 10 μL of the sample were applied in standard injection mode directly on Eksigent C8-CL-120 (3 μm 120 Å, 0.5 × 100 mm) column maintained at 40 °C. The mobile phase consisted of water with 0.1% formic acid (A) and ACN with 0.1% formic acid (B). Gradient profile under 10 μL/min constant flow conditions started from 95% A for one minute, followed by 60% A after 40 min and 5% in 45 min and maintained till 50 minute of the run. The column equilibration, under the initial gradient conditions was applied from the 50.1 to the 53 min. and as a pre-run for one minute before next injection. MS/MS detection was made using IDA method composed of mixed positive ionization scan modes and IDA criteria for dynamic m/z filtering. The method was constructed as follows: EMS, ER, IDA criteria and EPI. General survey EMS scan parameters covered the mass range 500–1400 Da with scan speed 4000 Da/s and 50–70 V DP values. ER scan was working in 250 Da/s scan rate (DP = 50–70 V) and was applied for the peptide charge determination. EPI scan was working in the range 50–1600 m/z (DP = 60 V and optimized rolling collision energy) and was used for mass spectra collection for the peptide sequence analysis. The ion source parameters were as follows: CUR: 25.00; TEM: 400.00; GS1: 20.00; GS2: 40.00; ihe: ON; IS: 5000.00; CAD: High. The most important IDA criteria used for selective m/z filtering were as follows: dynamic background subtraction – on, choose 1 to 3 most intense peaks from range 500–1400 m/z which charge state is 2–4 (include unknown) and exceeds 10,000 counts intensity, exclude former target ions for 30s after 3 occurrences and use enhanced resolution scan to confirm charge state.

### 2.9. Database searches

The Protein Pilot v 4.0.8 software (AB Sciex, USA), coupled with the MASCOT search engine v2.3 was used for the database searches. The data were searched against the *Zea mays* (212 248 sequences) database extracted from NCBI non-redundant database (version 05.2013). Mascot MS/MS ion searches were performed using trypsin as the protein-digesting enzyme, up to two missed cleavages were tolerated, and the following variable modifications were applied: Acetyl (N-term), Carbamidomethyl (C), Carbamyl (N-term), Deamidated (NQ), Formyl (N-term), Gln to pyro-Glu (N-term Q), Glu to pyro-Glu (N-term E) and Oxidation (M). The searches were conducted with a peptide mass tolerance of 0.7 Da and a fragment ion mass tolerance of 0.3 Da.

The proteins identified and unidentified by MASCOT searches were further processed using a BLAST search against the NCBI non-redundant protein sequence database (total number of sequences = 38 633 935) using the DELTA-BLAST algorithm (Domain Enhanced Lookup Time Accelerated BLAST) to confirm or define the probable function of the protein.

## 3. Results and discussion

Our previous results showed that the priming methods with simultaneous application of MEL were highly effective in improving cucumber and corn seed quality (Posmyk et al., 2008; Posmyk et al., 2009a,b; Janas et al., 2009). In fact, beneficial effects of priming were not visible in physiological tests performed under optimal conditions. Only when the plants were subjected to stress differences between the untreated and conditioned variants were they apparent. Our previous results (Janas et al., 2009) were very interesting when the corn seeds were germinated at 10 °C. The control non-primed seeds germinated after 5 days of imbibition and achieved ~97% germability after 12 days of experiments, whereas those treated with MEL reduced the lag phase up to 2 days and all seeds were germinated on 10th day of experiment. Hydropriming without MEL also reduced the lag phase to 3 days of imbibition but germinability of the seeds decreased to ~90% (Janas et al., 2009). The seed pre-sowing treatments accelerated germination rates of corn seeds at suboptimal temperature. In the presented research

**Table 1**
Identyfication of the proteins unique to different seed variants: control, non-treated (A), hydroprimed (B), all primed (B, C, D), all hydroprimed with melatonin (C, D) and characteristic of the seeds treated with 50 μM (C) or 500 μM (D) melatonin. All seeds ware germinated/incubated for 14 days at 5 °C. The Table contains: spots excised from gels and numbered by PDQuest Software used for gel qualitative analysis; function category consistent with *Nature* (Bevan et al., 1998); protein familys, names and accession numbers consistent with NCBI database; Mascot scor, sequence coverage (%), number of matched/unmatched (+/−) peptides, MW [kDa] and pI values.

| Spot N° | Function category | Protein family | Protein name | Accession N° | Mascot Scor | % Cover | Peptides +/− | MW [kDa] | pI |
|---|---|---|---|---|---|---|---|---|---|
| Protein unique to control, non-primed seeds (0)-A | | | | | | | | | |
| Proteins unique to hydroprimed seeds (3)-B | | | | | | | | | |
| Energy | | | | | | | | | |
| **8601** | 02.01 02 | TIM phosphate binding | Fructose-bisphosphate aldolase, cytoplasmic isozyme [*Zea mays*] | gi\|414879138 | 150 | 13 | 5/3 | 38.44 | 6.96 |
| Cell growth/division | | | | | | | | | |
| **6004** | 03.01 | NDPk | Hypothetical protein ZEAMMB73_585316 [*Zea mays*] 99% identities to Nucleoside diphosphate kinase I [*Zea mays*] | gi\|414867769 | 88 | 36 | 3/1 | 16.53 | 6.3 |
| Disease/Defence | | | | | | | | | |
| **3002** | 11.06 | α−crystallin-Hsps | 16.9 kDa class I heat shock protein 1 [*Zea mays*] | gi\|414876444 | 116 | 9 | 2/1 | 17.05 | 6.77 |
| Proteins unique to all primed seeds (5)-B, C, D | | | | | | | | | |
| Cell growth/division | | | | | | | | | |
| **6001** | 03.01 | SMP, LEA | Late embryogenesis abundant protein EMB564 [*Zea mays*] | gi\|22271 | 51 | 28 | 2/0 | 9.68 | 6.6 |
| Protein destination and storage | | | | | | | | | |
| **0401** | 06.13 | UBA like SF | Nascent polypeptide-associated complex α-subunit [*Zea mays*] | gi\|219363641 | 150 | 14 | 2/2 | 22.82 | 4.29 |
| Disease/Defence | | | | | | | | | |
| **1903** | 11.05 | PLN03020 | Low-temperature-induced 65 kDa protein [*Zea mays*] | gi\|413933891 | 79 | 5 | 4/1 | 58.72 | 4.75 |
| **4403** | 11.05 | SDR | General stress protein 39 [*Zea mays*] | gi\|414590804 | 219 | 14 | 7/4 | 38.60 | 9.06 |
| **1902** | 11.06 | Thioredoxin like | Protein disulfide isomerase [*Zea mays*] | gi\|625148 | 96 | 8 | 4/2 | 57.06 | 5.24 |
| Proteins unique to seeds hydroprimed with melatonin (6)−C, D | | | | | | | | | |
| Cell growth/division | | | | | | | | | |
| **8302** | 03.01 | SMP, LEA | Late embryogenesis abundant protein MGL3 [*Zea mays*] | gi\|413946305 | 93 | 14 | 3/2 | 22.75 | 8.8 |
| Transcription | | | | | | | | | |
| **6502** | 04.19 | WD40 | Guanine nucleotide-binding, β subunit-like protein [*Zea mays*] | gi\|413946394 | 76 | 20.4 | 3/0 | 36.21 | 6.13 |
| Protein synthesis | | | | | | | | | |
| **5103** | 05.07 | S1 like | Eukaryotic translation initiation factor 5A [*Zea mays*] | gi\|414887439 | 84 | 7 | 1/1 | 17.49 | 5.61 |
| Protein destination and storage | | | | | | | | | |
| **2102** | 06.20 | Cupin 2 | Vicilin-like embryo storage protein [*Zea mays*] | gi\|414872020 | 54 | 38.5 | 2/0 | 64.86 | 6.39 |
| Disease/defence | | | | | | | | | |
| **1001** | 11.06 | Glo EDI BRP like | Glyoxalase family protein superfamily [*Zea mays*] | gi\|414866129 | 51 | 18 | 2/0 | 15.07 | 5.47 |
| Unclassified | | | | | | | | | |
| **4402** | 13 | DUF1264 | Secreted protein [*Zea mays*] | gi\|413951152 | 164 | 27 | 6/3 | 27.33 | 5.84 |
| Proteins unique to seeds hydroprimed with 50 μM melatonin (2)−C | | | | | | | | | |
| Energy | | | | | | | | | |
| **7504** | 02.16 | AKRs | Aldose reductase [*Zea mays*] | gi\|413945705 | 115 | 7 | 3/2 | 35.64 | 6.47 |
| Protein destination and storage | | | | | | | | | |
| **3205** | 06.20 | Cupin 2 | Globulin−1S allele precursor [*Zea mays*] | gi\|195658011 | 162 | 11 | 5/4 | 49.93 | 6.16 |
| Proteins unique to seeds hydroprimed with 500 μM melatonin (5)-D | | | | | | | | | |
| Cell growth/division | | | | | | | | | |
| **0402** | 03.01 | SMP | Rab28 protein [*Zea mays*] | gi\|414864900 | 346 | 28 | 10/8 | 28.39 | 4.54 |
| **2401** | 03.01 | SMP, LEA | Late embryogenesis abundant protein D-34 [*Zea mays*] | gi\|414872767 | 134 | 10 | 3/2 | 27.16 | 5.41 |
| **9302** | 03.01 | SMP, LEA | Late embryogenesis abundant protein, group 3 [*Zea mays*] | gi\|414880812 | 51 | 10 | 2/1 | 23.53 | 8.96 |
| Transcription | | | | | | | | | |
| **0703** | 04.19 | RING | RING zinc finger domain superfamily protein [*Zea mays*] | gi\|414585686 | 56 | 3 | 1/1 | 42.82 | 5.77 |
| Protein destination and storage | | | | | | | | | |
| **2702** | 06.01 | Hip | Hsc70-interacting protein [*Zea mays*] | gi\|552562419 | 142 | 13 | 5/3 | 43.36 | 5.06 |

**Table 2**
Proteins showing different expression in axes of the control nt seeds (A) germinated/incubated for 14 days at 5 °C, to those from all hydroprimed seeds: H, HMel50, HMel500 (B, C, D). The Table contains: spots excised from gels and numbered by PDQuest Software used for quantitative gel analysis; function category consistent with *Nature* (Bevan et al., 1998); protein names and accession numbers consistent with NCBI database; Mascot scor, sequence coverage (%), number of matched/unmatched (+/−) peptides, MW [kDa] and pI values; and relative abundance fold change in comparision to control (A) seeds − statistical significance of the differences (at $p \leq 0.01$) between the compared variants is marked by bolded font of numbers (the cut-off for differentially regulated proteins is a 5-fold change).

| Spot N° | Function category | Protein name | Accession N° | Mascot Scor | % Cover | Peptides +/− | MW [kDa] | pI | Fold change $p \leq 0.01$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | A/B | A/C | A/D |
| Proteins up-regulated in A in comparision to B, C, D. | | | | | | | | | | | |
| Energy | | | | | | | | | | | |
| **4601** | 02.01 | Cytosolic 3-phosphoglycerate kinase [*Zea mays*] | gi\|28172917 | 180 | 27 | 8/6 | 31.61 | 5.01 | 0.91 | 1.87 | **5.45** |
| **3702** | 02.01 02 | Enolase [*Zea mays*] | gi\|195619804 | 111 | 14 | 5/2 | 48.13 | 5.7 | 2.66 | **11.5** | **18.5** |
| Cell growth/division | | | | | | | | | | | |
| **8802** | 03.01 | Embryonic protein DC-8-like [*Zea mays*] | gi\|413936531 | 210 | 13 | 7/3 | 61.73 | 7.27 | 1.37 | **6.90** | 1.70 |
| Protein destination and storage | | | | | | | | | | | |
| **3302** | 06.20 | Germin-like protein 2 [*Zea mays*] | gi\|413956703 | 241 | 7 | 5/5 | 71.09 | 6.31 | 1.29 | **8.71** | 2.52 |
| Transporters | | | | | | | | | | | |
| **1301** | 07.01 | High-affinity potassium transporter 3 [*Zea mays*] | gi\|576866892 | 89 | 16 | 3/2 | 16.69 | 4.86 | 3.46 | **20.1** | 2.59 |
| Disease/defence | | | | | | | | | | | |
| **7301** | 11.06 | 1-Cys peroxiredoxin antioxidant [*Zea mays*] | gi\|87133468 | 866 | 48 | 50/21 | 24.89 | 6.31 | 2.15 | **6.49** | 1.44 |
| Unclassified | | | | | | | | | | | |
| **2202** | 13 | Hypothetical protein ZEAMMB73_452374 [*Zea mays*] | gi\|413916092 | 76 | 15 | 3/1 | 16.13 | 4.98 | 1.95 | **7.09** | 2.35 |
| Proteins down-regulated in A in comparision to B, C, D. | | | | | | | | | | | |
| Energy | | | | | | | | | | | |
| **5301** | 02.01 | Triosephosphate isomerase, cytosolic [*Zea mays*] | gi\|414876338 | 149 | 14 | 4/3 | 27.01 | 5.52 | 0.25 | **0.10** | 0.60 |
| **6603** | 02.01 02 | Glyceraldehyde-3-phosphate dehydrogenase 2 [*Zea mays*] | gi\|6016075 | 96 | 4 | 1/1 | 36.49 | 6.46 | **0.19** | **0.08** | |
| **3301** | 02.20 | Ferritin [*Zea mays*] | gi\|29840836 | 123 | 15 | 5/3 | 31.43 | 6.13 | | **0.04** | **0.04** |
| Protein synthesis | | | | | | | | | | | |
| **3101** | 05.04 | Eukaryotic translation initiation factor 5A [*Zea mays*] | gi\|414887439 | 78 | 7 | 1/1 | 17.49 | 5.61 | **0.05** | **0.003** | **0.04** |
| Protein destination and storage | | | | | | | | | | | |
| **1802** | 06.01 | Protein disulfide isomerase [*Zea mays*] | gi\|145666464 | 96 | 10 | 5/3 | 56.83 | 5.01 | 0.21 | **0.18** | **0.18** |
| **3801** | 06.01 | Chaperonin HSP 60 [*Zea mays*] | gi\|22242 | 245 | 15 | 9/5 | 61.26 | 5.68 | **0.08** | 0.21 | 0.55 |
| **4001** | 06.20 | Globulin–1S allele precursor [*Zea mays*] | gi\|195658011 | 240 | 10 | 6/4 | 49.89 | 6.16 | **0.02** | **0.05** | **0.06** |
| Disease/defence | | | | | | | | | | | |
| **7102** | 11.05 | Class II heat shock protein [*Zea mays*] | gi\|413939226 | 269 | 50 | 10/7 | 18.34 | 6.6 | **0.03** | **0.02** | **0.06** |
| **7506** | 11.05 | Hypothetical protein ZEAMMB73_578099, [*Zea mays*] 78% identity to Stress responsive protein [*Zea mays*] | gi\|414866592 | 302 | 23 | 12/10 | 38.37 | 6.3 | **0.02** | **0.02** | **0.13** |
| **1102** | 11.06 | Peroxiredoxin-5 [*Zea mays*] | gi\|413950901 | 58 | 6 | 1/1 | 17.31 | 4.85 | **0.10** | **0.25** | **0.58** |
| **2001** | 11.06 | Glyoxalase family protein superfamily [*Zea mays*] | gi\|414866129 | 86 | 13 | 2/1 | 15.07 | 5.47 | **0.04** | **0.04** | **0.04** |
| **2501** | 11.06 | Glyoxalase family protein isoform II [*Zea mays*] | gi\|413917004 | 56 | 6 | 2/1 | 32 | 5 | **0.07** | 0.38 | **0.13** |
| **3401** | 11.06 | Glyoxalase I [*Zea mays*] | gi\|37932483 | 145 | 17 | 8/3 | 32.34 | 5.59 | **0.10** | 0.21 | 0.39 |

the seeds were subjected to a stress temperature 5 °C (below minimum for maize germination) and the influence of different types of priming on the embryo proteome was verified. For this purpose, proteomes of embryos isolated from nt, H, HMel50 and, HMel500 seeds incubated for 14 days at 5 °C were compared. The first two seed variants (nt and H) were the control/reference for the determination of MEL-associated proteins, because it is known that already hydropriming with water generates changes in proteome of seeds (Gallardo et al., 2001; Wu et al., 2011).

Protein maps produced in four independent replicates showed a high level of reproducibility. PDQuest software was able to recognize and marked as corresponding ~95% of the visualised protein spots in the replicated gel images. The data obtained from densitometric analysis of PDQuest master gels were the basis for qualitative (Table 1) and quantitative (Tables 2 and 3) analyses of different experimental seed variants. 78 protein spots were marked on gel A—representing the proteome of axes from nt seeds, 86 on gel B—proteome of H seed variant, 90 on gel C—proteome of HMel50 seed variant, and 94 on gel D—proteome of HMel500 seed variant (Figs. 1 and 2). Already the first data suggested general repression of protein biosynthesis in response to low temperature stress. In comparison to the previous work, the quantity of protein spots in particular seed variants decreased by about 50% (the spot numbers in particular gels after seed germination under optimal temperature conditions, at 25 °C for 24 h were as follows: A—130, B—143, C—180, D—160; data in press). Despite significant slowdown in seed metabolism caused by low temperature, hydropriming and especially hydropriming supplemented with MEL stimulated biosynthesis of new proteins in the seed embryonic axes under stress conditions (Fig. 2).

**Table 3**
Proteins showing different expression in axes of the hydroprimed seeds (B) germinated/incubated for 14 days at 5 °C, to those from all seeds hydroprimed with melatonin: HMel50, HMel500 (C, D). The Table contains: spots excised from gels and numbered by PDQuest Software used for quantitative gel analysis; function category consistent with *Nature* (Bevan et al., 1998); protein names and accession numbers consistent with NCBI database; Mascot scor, sequence coverage (%), number of matched/unmatched (+/−) peptides, MW [kDa] and pI values; and relative abundance fold change in comparision to hydroprimed (B) seeds-statistical significance of the differences (at $p < 0.01$) between the compared variants is marked by bolded font of numbers (the cut-off for differentially regulated proteins is a 5-fold change).

| Spot N° | Function category | Protein name | Accession N° | Mascot Scor | % Cover | Peptides +/− | MW [kDa] | pI | Fold change $p \leq 0.01$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | B/C | B/D |
| Proteins up-regulated in B in comparision to C, D | | | | | | | | | | |
| Energy | | | | | | | | | | |
| **4601** | 02.01 | Cytosolic 3-phosphoglycerate kinase [*Zea mays*] | gi\|28172917 | 247 | 27 | 8/6 | 31.61 | 5.01 | 2.05 | **5.96** |
| **2703** | 02.01 02 | Enolase1 [*Zea mays*] | gi\|298544549 | 418 | 28 | 11/8 | 48.11 | 5.14 | 1.87 | **8.36** |
| **3702** | 02.01 02 | Enolase [*Zea mays*] | gi\|195619804 | 111 | 14 | 5/2 | 48.13 | 5.7 | 4.33 | **6.95** |
| Cell growth/division | | | | | | | | | | |
| **8802** | 03.01 | Embryonic protein DC-8-like [*Zea mays*] | gi\|413936531 | 210 | 13 | 7/3 | 61.73 | 7.27 | **5.04** | 1.25 |
| Protein destination and storage | | | | | | | | | | |
| **3302** | 02.20 | Germin-like protein 2 [*Zea mays*] | gi\|413956703 | 241 | 7 | 5/5 | 71.09 | 6.31 | **6.76** | 1.96 |
| **3801** | 06.01 | Chaperonin hsp60 [*Zea mays*] | gi\|22242 | 245 | 15 | 9/5 | 61.26 | 5.68 | 2.60 | **6.88** |
| Disease/defence | | | | | | | | | | |
| **4403** | 11.05 | General stress protein 39 SDR family protein [*Zea mays*] | gi\|414590804 | 219 | 14 | 7/4 | 38.60 | 9.06 | 4.00 | **25.5** |
| **7506** | 11.05 | Stress responsive protein [*Zea mays*] | gi\|414866592 | 302 | 23 | 12/10 | 38.37 | 6.3 | 1.15 | **7.10** |
| **1102** | 11.06 | Peroxiredoxin-5 [*Zea mays*] | gi\|413950901 | 58 | 6 | 1/1 | 17.31 | 4.85 | 2.63 | **5.98** |
| **2501** | 11.06 | Glyoxalase family protein isoform 2 [*Zea mays*] | gi\|413917004 | 56 | 6 | 2/1 | 32.34 | 5.59 | **5.59** | 1.88 |
| Proteins down-regulated in B in comparision to C, D | | | | | | | | | | |
| Energy | | | | | | | | | | |
| **8502** | 02.01 02 | Glyceraldehyde-3-phosphate dehydrogenase1 cytosolic isoform X1 [*Zea mays*] | gi\|413921395 | 56 | 13 | 5/1 | 30.34 | 6.32 | **0.17** | |
| Cell growth/division | | | | | | | | | | |
| **6001** | 03.01 | Embrygenic protein [*Zea mays*], Late embryogenesis abundant protein EMB564 [*Zea mays*] | gi\|22271 | 51 | 28 | 2/0 | 9.68 | 6.6 | **0.03** | **0.02** |
| Protein synthesis | | | | | | | | | | |
| **3101** | 05.04 | Eukaryotic translation initiation factor 5A [*Zea mays*] | gi\|414887439 | 78 | 7 | 1/1 | 17.49 | 5.61 | **0.06** | 0.75 |
| Protein destination and storage | | | | | | | | | | |
| **3001** | 06.20 | Vicilin-like embryo storage protein [*Zea mays*] | gi\|22287 | 54 | 2 | 1/1 | 60.20 | 9.33 | **0.08** | **0.15** |
| Disease/defence | | | | | | | | | | |
| **1903** | 11.05 | Low-temperature-induced 65 kDa protein [*Zea mays*] | gi\|413933891 | 79 | 5 | 4/1 | 58.72 | 4.75 | **0.07** | 0.80 |

### *3.1. Hydropriming influence on axes proteome in stressed corn seeds*

Proteomic analysis of embryo extracts isolated from nt, H, HMel50 and HMel500 seeds allowed us to identify proteins that appeared after seed priming (only in gels B, C, D—Table 1, Fig. 1). There were the proteins associated mainly with stress conditions *i.e.*: low-temperature-induced 65 kDa protein—1903; general stress protein 39–4403; protein disulfide isomerase (PDIa)—1902 and also late embryogenesis abundant (LEA) protein EMB564–6001 (Table 1, Fig. 1BCD).

Initially small hydrophilic plant seed proteins LEA were implicated in embryogenesis in higher plant seeds (Dure et al., 1981); they appeared during late stages of embryogenesis under physiological seed desiccation. However, the function of these proteins is not clear but they are often implicated in the plant abiotic stresses response, especially if stress limits water absorption. They protect other proteins from aggregation due to dehydration or osmotic stresses associated with low temperature (Goyal and Walton, 2005). LEA proteins are particularly protective of mitochondrial membranes against structural damage (Tolleter et al., 2010).

PDIa are a family of redox active proteins containing a conserved structural disulfide in the redox-inactive thioredoxin-like domain. They are involved in oxidative protein folding in the endoplasmic reticulum acting as catalysts and folding assistants. They catalyze the formation of disulfide bonds in new polypeptides, but they also exhibit reductase activity acting as isomerases to correct any non-native disulfide bonds. Moreover, they can act as chaperones to prevent protein aggregation and facilitate folding of new proteins.

Another protein – nascent polypeptide-associated complex α-subunit protein (NACA; 0401) – found in the conditioned seeds (Table 1, Fig. 1B–D) also appears to be involved in the improvement of protein destination, modifications or degradation. In complex with β-subunit (NACB) NAC is known as a basal transcription factor 3 (Btf3), which plays a crucial role in protection of newly formed proteins from interaction with inappropriate cytosolic factors (Panasenko et al., 2009). NAC directly interacts with a signal recognition particle and was reported to be involved in new protein translocation to the endoplasmic reticulum to prevent their mistargeting (Lauring et al., 1995). Expression of NAC was differentially regulated by abiotic stresses, including salinity, drought and cold. Constitutive over-expression of NAC in *Arabidopsis* was observed during normal growth and it enhanced tolerance to salt and drought stresses. NAC protects newly synthesized polypeptides involved in the mechanisms allowing plants to tolerate abiotic stress-induced damage, thus preventing cell death (Karan and Subudhi, 2012).

In the embryos isolated from H seeds an additional 16.9 kDa I class heat shock protein (HSP; 3002), similar to nucleoside diphos-

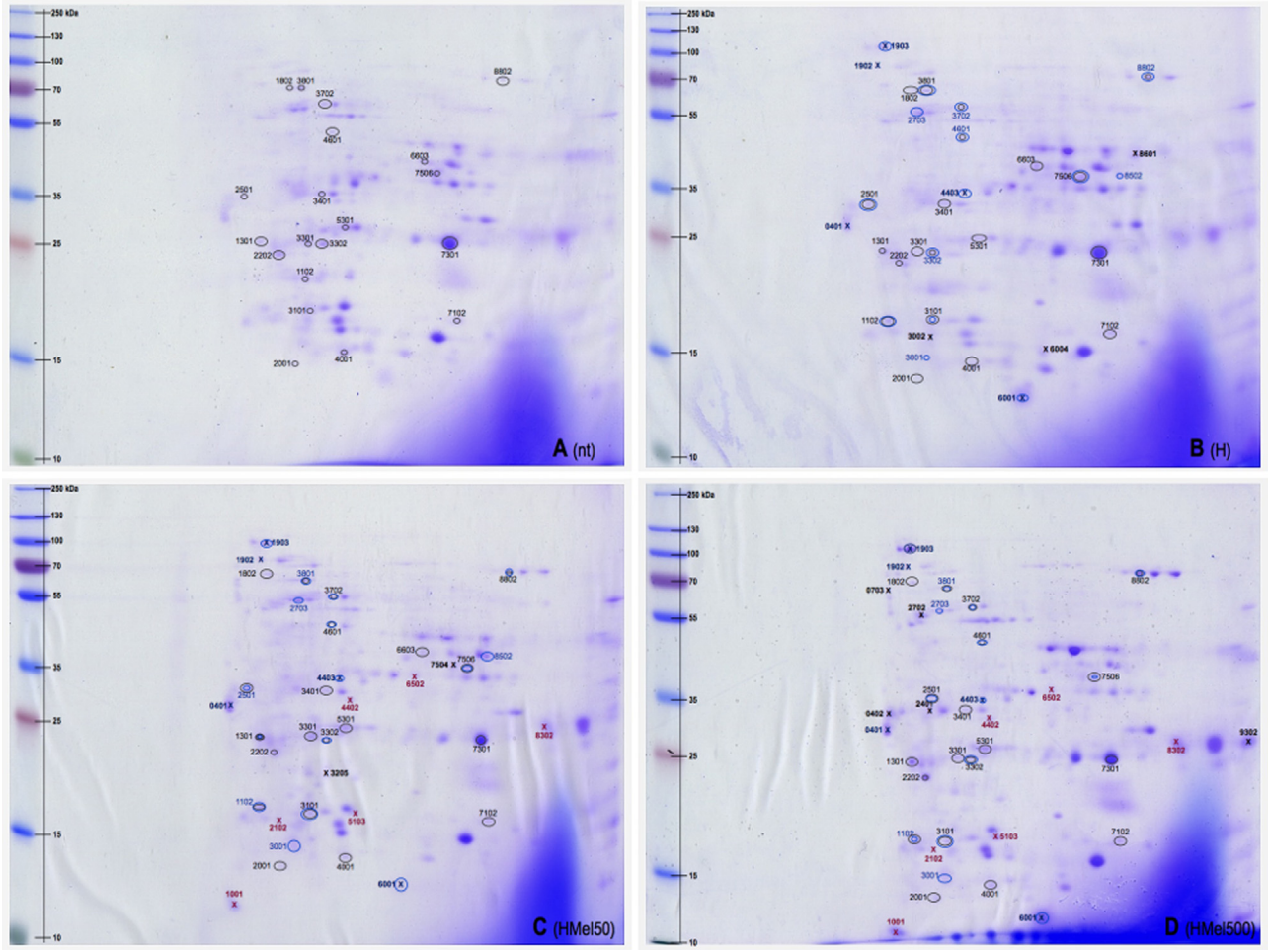



**Fig. 1.** Analytical gels of IEF/SDS-PAGE (2D) separation of proteins from: control nt (A), hydroprimed (B), hydroprimed with MEL 50 μM (C) and 500 μM (D) seeds germinated in darkness at 5 °C for 14 days. The proteins were separated by first dimensional pH 3–10 non-linear IPG strips and 12% vertical slab gels in the second dimension (ImperialTM Protein Stain). The proteins were numbered (PDQuest Software, BioRad) in a preparative 2D electrophoresis gel and excised for MS/MS analysis, corresponding to the spot/proteins in Tables 1–3. According to the qualitative analysis the black X symbols mean proteins characteristic only of particular gels (A or B or C or D), blue X symbols mean proteins characteristic of all hydroprimed seeds (BCD), red X symbols means proteins characteristic of seeds hydroprimed with MEL (CD) (see Table 1). According to the quantitative analysis ellipses are used to mark proteins five times up- or down- regulated: black for comparison of A with B–D (see Table 2), blue for comparison of B with C and D (see Table 3).

phate kinase I protein (NDPk; 6004) and fructose-bisphosphate aldolase, cytoplasmic isozyme (8601) were observed (Table 1, Fig. 1B). First class cytoplasmic LMW HSPs, with molecular mass 15–30 kDa, are the major proteins synthetized during heat stress, but they also frequently accumulate in seeds, seed pods, and flowers during a normal growing season (Hernandez and Vierling, 1993). The clear function of LMW HSPs is unknown but their evolutionary conservation and presence in different compartments (cytoplasma, endomembrane and chloroplasts) suggest that they are significant for plant survival.

NDPk domains are present in a large family of structurally and functionally conserved proteins from bacteria to humans that generally catalyze the transfer of gamma-phosphates of a NTP donor onto a nucleoside diphosphate NDP acceptor through a phosphohistidine intermediate. Starting with ADP and GTP, the activity of NDPk produces GDP and ATP. Thus, with fructose-bisphosphate aldolase – glycolytic enzymes, which catalyze decomposition of fructose 1,6-bisphosphate into triose phosphates dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, NDPk could be involved in energy acquisition and distribution in cell.

There were no proteins unique to the control nt seeds (gel A) (Table 1, Fig. 1A), however, some enzymes of primary metabolism were overexpressed in them in comparison with the primed seed

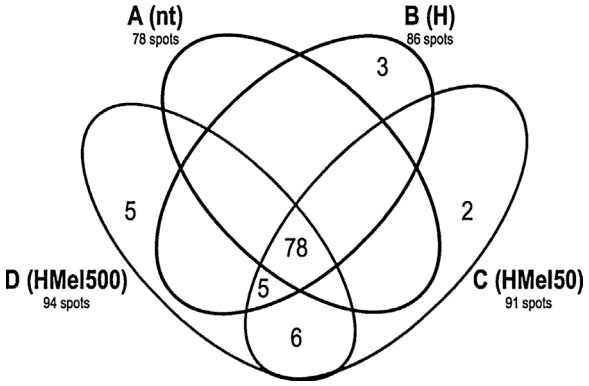



**Fig. 2.** Venn diagram analysis of the proteins accumulated in the embryonic axes of various seed variants germinated in darkness at 5 °C for 14 days. The number of proteins in axes isolated from the control non-treated (A), hydroprimed (B), hydroprimed with MEL 50 μM (C) and 500 μM (D) seeds, are shown in the respective segments.

variants (gels B, C, D) *i.e.*: cytosolic 3-phosphoglycerate kinase (4601) and enolase (3702) (Table 2, Fig. 1A). However others, such as cytosolic triosephosphate isomerase (5301), glyceraldehyde-

3-phosphate dehydrogenase 2 (6603) and ferritin (3301) were overexpressed in the conditioned seeds (Table 2, Fig. 1B–D). The control nt seeds were characterized by greater quantity of embryonic DC-8-like protein (8802) (Table 2, Fig. 1A). In fact its level gradually decreased during the first steps of imbibition of maize seeds (Tnani et al., 2012) and in the case of hydroprimed seeds these steps took place during the first phase of priming – hydration. Moreover, in the embryos of nt seeds, greater quantities of germin-like protein 2 (3302) and 1-Cys peroxiredoxin (7301) were synthetized (Table 2, Fig. 1A). 1-Cys peroxiredoxin is a member of the peroxiredoxin superfamily. It can protect cells against membrane oxidation through GSH-dependent reduction of phospholipid hydroperoxides to corresponding alcohols. In higher plants, members of this antioxidant group have previously been isolated from monocotyledons. It has been suggested that seed peroxiredoxins protect tissues from ROS and RNS during desiccation and early imbibition and/or are involved in the protection maintenance during dormancy (Haslekas et al., 1998). Germins and germin-like proteins are a plant cupin subfamily of water-soluble glycoproteins. Germin was first identified during wheat germination (Thompson and Lane, 1980). There is evidence suggesting that GLPs play a crucial role not only in plant development, but also in plant defense responses (Lane 2002). This was shown by increased expression of certain *GLPs* in various plants under stresses such as fungal, bacterial, and viral infections (Zimmermann et al., 2006), parasite attacks, insect invasions (Lou and Baldwin, 2006), chemical toxicities, salt pressures (Hurkman et al., 1994) and drought stresses (Ke et al., 2009). Moreover, enhanced resistance of transgenic *GLP* plants to various stresses was noticed.

Another protein belonging to the cupin family—globulin–1 S allele precursor (4001), was overexpressed in the primed seeds (Table 2, Fig. 1BCD). Moreover, improvement of protein folding, destination and modifications in the primed seeds was possible by overexpression of other proteins disulphide isomerase isoenzym (1802) and chaperonin HSP 60 (3801). In gels B, C and D significant overexpression of 6 defence proteins *i.e.*: HSP class II (7102), stress responsive protein (7506), peroxiredoxin-5 (1102) and 3 of glyoxalases (2001, 2501, 3401), was visualised (Table 2, Fig. 1BCD).

Plant peroxiredoxins are thiol-based peroxide reductases with wide substrate specificity, ranging from hydrogen peroxide to alkyl hydroperoxides and peroxinitrite. Prx have several features in common (Dietz 2011). They are numerous proteins that are routinely detected in proteomics research. They interact with other proteins *i.a* glutaredoxins, thioredoxins and cyclophilins as reductants, but also non-dithiol-disulphide exchange proteins. In studies using transgenic plants, peroxiredoxins were shown to influence metabolic integrity to protect DNA from damage *in vitro* and *in vivo*, and to modulate intracellular signaling related to ROS and RNS.

The results of numerous recent studies have shown that alleviation of oxidative damage and increased resistance to abiotic stresses are often correlated with more efficient antioxidative and glyoxalase systems. Stress-induced Gly I protein and Gly II mRNA expression was first demonstrated by Espartero et al. (1995) in tomato seedlings subjected to NaCl, mannitol and ABA treatment. The regulatory roles of the glyoxalase system and ROS detoxification systems in plant abiotic stress tolerance have increasingly attracted interest because excessive ROS and methylglyoxal production is a common repercussion of both abiotic and biotic stresses in plants (Hossain et al., 2011). Plants have a complex network of enzymatic and non-enzymatic scavenging pathways or detoxification systems that cooperate to efficiently counteract the harmful effects of ROS and MG as well as to perform their signaling function. In plants, MG is detoxified mainly *via* the glyoxalase system. The glyoxalase system could also play a role in maintaining glutathione homeostasis by recycling its reduced form that would be affected noenzymatically by MG to form hemithioacetal. In addition, in plants, ROS levels are controlled *via* a universal antioxidant network. The interplay between ROS and components of the antioxidant and glyoxalase systems could generate compartment-specific changes in both the absolute concentrations of ROS, MG and antioxidant compounds as well as in the ascorbate and glutathione redox ratios. Under stress conditions, these redox signals could interfere with the signaling networks complementary to the antioxidant system and regulate defense gene expression, thus coordinating the necessary readjustments in the redox-regulated plant defense to overcome oxidative stress (Hossain et al., 2011).

### 3.2. Melatonin influence on axes proteome in stressed corn seeds

MEL application during the hydropriming procedure resulted in an additional protein biosynthesis. One of them was the earlier described protective dehydrin—LEA protein MGL3 (8302), and the other was glyoxalase family protein (1001) (Table 1, Fig. 1CD). The glyoxalase system is an enzyme set responsible for detoxification of MG and the other reactive aldehydes. They are produced as a normal part of metabolism and especially under abiotic stress conditions and they were discussed earlier in this paper.

Moreover, an additional cupin: vicilin-like embryo storage protein (2102) was also noticed (Table 1, Fig. 1CD). Vicilin is a globulin which occurs mainly in legume seeds. This family contains 11S and 7S plant germins and seed storage proteins, the latter provide the major nitrogen source for developing plants.

In the MEL-treated seeds a secreted protein (4402) of unknown function appeared (Table 1, Fig. 1CD). DUF1264 family contains a number of bacterial and eukaryotic proteins of unknown function that are approximately 200-residue long. Some of this family's members are putative lipoproteins, but it is still unclear why they appear.

In comparison to the nt and H seeds, in the MEL-treated ones additional elements involved in protein synthesis destination and storage were observed (*i.e.*: guanine nucleotide-binding, β subunit-like protein—6502; eukaryotic translation initiation factor 5A—5103) (Table 1, Fig. 1CD). Guanine nucleotide binding proteins (G proteins) are a family of membrane-associated ones that couple extracellularly-activated integral-membrane receptors to intracellular effectors, such as ion channels and enzymes, and they are responsible for second messenger molecule concentration (Simon et al., 1993). G proteins are usually composed of 3 subunits (α, β and γ) which, in the resting state, associate as trimers at the inner face of the plasma membrane. GDP is attached to the α subunit. Stimulation of the G protein by an active receptor leads to its conversion into GTP. This results in disconnection of the α from the β and γ subunits, which always remain closely associated β-γ dimers. Both the α and β-γ subunits are able to interact with effectors, either individually or in a cooperative mode. The intrinsic GTPase activity of the α subunit hydrolyses GTP to GDP. This makes the α subunit return to its inactive conformation and allows it to reassociate with the β-γ subunit, thus rebuilding the system in its basic state. Although originally the α subunit was thought to be a passive attenuator and membrane anchor for the β-γ subunit, now it is recognized as playing an active role in a number of different G protein-coupled signaling events (Clapham and Neer 1993). G proteins have been shown to change the activity of some isoforms of adenylyl cyclase, phospholipase C and some ion channels. They are involved in phosphorylation of receptors *via* specific kinases and have been implicated in the p21ras-dependent activation of the MAP kinase cascade. They are also likely to contribute to the recognition of specific receptors and are associated with a variety of functions ranging from signal transduction and transcription regulation to control of cell cycle, apoptosis and autophagy (Stirnimann et al., 2010).

Eukaryotic translation Initiation Factor 5A contains an S1-like RNA-binding domain. It is an evolutionarily conserved protein discovered in eukaryotes. eIF5A is the only protein that has the unusual amino acid-hypusine. Hypusine is a post-translationally modified lysine and it is essential for eIF5A functioning. eIF5A in a hypusine-dependent manner interacts with components of 80S ribosome and translation elongation factors 2, thus eIF5A plays a role in translation elongation instead of translation initiation as previously proposed. This superfamily also contains the cold shock domain, which is a homolog of the S1 domain. Both domains are members of the oligonucleotide/oligosaccharide binding, which binds RNA.

In HMel50 seeds an additional isoenzym, aldose reductase (7504) and globulin–1S allele precursor (3205) were noticed (Table 1, Fig. 1C). The former improves energy metabolism under stress conditions, the latter belongs to seed storage proteins and constitutes nutrient reservoir. In the HMel500 seeds also additional Rab28 protein (0402), LEA D-34 (2401), and LEA group 3 (9302) were observed (Table 1, Fig. 1C). The role of LEA proteins in stresses combat has been previously described in this paper.

The Rab28 protein is known as a plant seed maturation protein but it should be underlined that it is ABA-responsive. The *rab28* gene responds to ABA treatment. This gene encodes a protein of 28 kDa molecular weight, which shows significant homology with the LEA D-34 protein determined in cotton (Pla et al., 1991). The *rab28* mRNA has been identified as ABA-inducible in seed embryos and young leaves. It is also activated by water-stress in leaves of wild-type plants. Inuction of the *rab28* gene was also studied in maize viviparous mutants (Pla et al., 1991). Thus, Rab28 proteins could be synthetized under stress conditions in response to elevated ABA level generated by stress. ABA is lived-in phytohormone that plays important roles during many phases of plant life cycle, including seed development, maturity and dormancy, and especially the acquisition of desiccation tolerance. Elucidation of the molecular basis of ABA-mediated plant response to stress is of interest not only in basic research on plant adaptation but also in applied research on plant productivity.

RING zinc finger domain superfamily protein (0703) and Hsc70-interacting protein (2702) were also characteristic of the embryos from HMel500 seeds (Table 1, Fig. 1D). Both proteins could be involved in protein turnover by ubiquitination. Ubiquitination is a unique, widely conserved molecular mechanism to degrade proteins (UPS). The covalent attachment of ubiquitin to a target substrate involves the sequential action of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). Usually, once a targeted protein has been labelled with a polyubiquityn chain, it is recognized and degraded by 26S proteasome. The E3 are the proteins/enzymes, which specifically select the target proteins ensuring the specificity of entire process. E3-mediated protein degradation regulates almost all cellular processes including the abiotic stress response (Lyzenga and Stone, 2012). The plant genomes examined thus far contain two or more E1 enzymes, dozens of E2s and a large number of E3s. Analysis of the *Arabidopsis* genome idicated two E1s, 37 E2s, and over 1300 E3 encoding genes (Kraft et al., 2005). Specificity of the UPS is managed mainly by the substrate-selecting E3 ubiquitin ligases. The large number of ubiquitin enzymes suggests that many cellular processes are regulated *via* protein ubiquitination (Stone, 2014). The majority of plant E3s are homologous to E6-associated carboxy-terminus, U-box, or RING E2 binding domain. It is known that SDIR1 is a RING finger E3 ligase that positively regulates stress-responsive ABA signaling in *Arabidopsis* (Zhang et al., 2007) and it has been proved that RING zinc finger protein ZmZF1 from maize confers salt and drought tolerance in transgenic *Arabidopsis* (Huai et al., 2009). Moreover, *A. thaliana* Carboxyl Terminus of Hsc70-Interacting Protein is a U-box-type E3 ligase named for its sequence similarity to mammalian co-chaperone CHIP which targets non-native or damaged proteins for degradation by the 26S proteasome (Murata et al., 2001). Since cold and heat stresses modulate expression of AtCHIP one would expect that E3 improves stress tolerance by targeting denatured and injured proteins for degradation. Thus, the aforementioned RING zinc finger domain superfamily protein (0703) and Hsc70-interacting protein (2702) in the embryos from HMel500 seeds could have an important function in stress signaling and response to cold stress.

Energetic metabolism enzymes (*i.e.*: cytosolic 3-phosphoglycerate kinase—4601; enolases—2703, 2702), stress proteins (*i.e.*: general stress protein 39 SDR family protein—4403; stress responsive protein—7506; peroxiredoxin-5–1102; glyoxalase family protein isoform 2—2501) a protective protein (chaperonin hsp60–3801) were observed in all hydroprimed seeds, but were overexpressed only in the variant HMel500 (Table 3, Fig. 1). The differences between H and HMel50 variants were not so great. Unquestionably the H variant contained much more germin-like protein 2 (3302) and embryonic protein DC-8-like (8802) then both MEL-treated ones (Table 3, Fig. 1).

On the other hand, the MEL-treated seeds (especially HMel50) showed overexpression of some proteins compared to H seeds *i.e.*: glyceraldehyde-3-phosphate dehydrogenase1 cytosolic isoform X1 (8502), LEA protein EMB564 (6001), eukaryotic translation initiation factor 5A (3101), vicilin-like embryo storage protein (3001) and low-temperature-induced 65 kDa protein (1903) (Table 3, Fig. 1). Their physiological roles were described earlier in this work.

## 4. Conclusions

Although protein biosynthesis was significantly inhibited under prolonged cold stress conditions (14 d/5 °C) positive changes in the embryo proteome were observed in the hydroconditioned seeds and especially in those hydroconditioned with MEL. To summarize (Fig. 2), hydroconditioning (seed variants: H, HMel50, HMel500) generated 5 new proteins in embryonic axes of seeds germinated at chilling temperature compared to the control nt ones. When this presowing treatment was supplemented with MEL application (seed variants: HMel50, HMel500) additional 6 specyfic proteins appeared. Moreover, 2 and 5 characteristic spots were noticed in HMel50 and HMel500 variants respectivelly.

MEL induced modifications improving the respiratory/energetic metabolism of the conditioned seeds, and these changes could be crucial for efficient stress amelioration. Moreover, in the seeds pretreated with MEL, many more stress-related, defense and detoxifying proteins were synthetized. Plants ability to survive abiotic stresses such as salinity, radiation, heavy metals, nutrient deprivation, cold and drought heavily depends on proteomic plasticity including: protein synthesis *de novo*, their posttranslational modifications and folding as well as destination to their activity sites and also their specific degradation and turnover. UPS plays a critical role allowing plants to alter their proteome in order to effectively and efficiently react to environmental stresses (Lyzenga and Stone, 2012). In the seeds hydroconditioned with MEL some characteristic proteins that could play E3 ligase role and extended UPS action were noticed. The plant response to adverse environmental conditions is a complex and coordinated process involving activation of signaling networks and changes in the expression of hundreds of genes. By modulating the amount of transcription factors, UPS may affect the changes in gene expression required to mitigate the potential negative effects of environmental stress.

All these above mentioned positive proteome modifications elicited by hydropriming and especially by MEL, which occurred even under harmful temperature conditions, could explain the phenomenon of better stress tolerance and also of more effective corn

seedling regeneration after stress alleviation observed by us previously (Janas et al., 2009).

To conclude, MEL seems to be a promising seed biostimulator. This was already evident after the physiological and biochemical tests (Posmyk et al., 2008; Posmyk et al., 2009a,b; Janas et al., 2009) but now the mechanism by which MEL improves seed quality and extend their tolerance to chilling stress was elucidated on the proteomic level. Moreover, MEL-induced changes of seed physiology may have preventive (preparation for the stress before it occurs – data in press), and/or emergency character (better tolerance of occurring stress – presented data), which certainly enhances crop yield under optimal and especially under unfavorable conditions. Future research should be extended to other species of important crops.

## Acknowledgments

Research work supported by the National Science Centre of Poland as NCN NN310 111940 project.

## References

Bajwa, V.S., Shukla, M.R., Sherif, S.M., Murch, S.J., Saxena, P.K., 2014. Role of melatonin in alleviating cold stress in *Arabidopsis thaliana*. J. Pineal Res. 56, 238–245.

Bevan, M., Bancroft, I., Bent, E., Love, K., Goodman, H., Dean, C., et al., 1998. Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. Nature 391, 485–488.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dry binding. Anal. Biochem. 72, 248–254.

Cho, M.H., No, H.K., Prinyawiwatkul, W., 2008. Chitosan treatments affect growth and selected quality of sunflower sprouts. J. Food Sci. 73, 70–77.

Clapham, D.E., Neer, E.J., 1993. New roles for G protein beta-gamma dimers in transmembrane signalling. Nature 365, 403–406.

Dietz, K.-J., 2011. Peroxiredoxins in plants and cyanobacteria. Antioxid. Redox. Sign. 15, 1129–1159.

Dure, L., Greenway, S.C., Galau, G.A., 1981. Developmental biochemistry of cottonseed embryogenesis and germination: changing messenger ribonucleic acid populations as shown by *in vitro* and *in vivo* protein synthesis. Biochemistry 7, 4162–4168.

Espartero, J., Aguayo, I.S., Pardo, J.M., 1995. Molecular characterization of glyoxalase-I from a higher plant; upregulation by stress. Plant Mol. Biol. 29, 1223–1233.

Gallardo, K., Job, C., Groot, S.P., Puype, M., Demol, H., Vandekerckhove, J., Job, D., 2001. Proteomic analysis of *Arabidopsis* seed germination and priming. Plant Physiol. 126, 835–848.

Goyal, K., Walton, L.J., 2005. Tunnacliffe A. LEA proteins prevent protein aggregation due to water stress. Biochem. J. 388, 151–157.

Haslekas, C., Stacy, R.A.P., Nygaard, V., Culiáñez-Macià, F.A., Aalen, R.B., 1998. The expression of a peroxiredoxin antioxidant gene, *AtPer1*, in *Arabidopsis thaliana* is seed-specific and related to dormancy. Plant Mol. Biol. 36, 833–845.

Hernandez, L.D., Vierling, E., 1993. Expression of low molecular weight heat-shock proteins under field conditions. Plant Physiol. 101, 1209–1216.

Hossain, M.A., Teixeira da Silva, J.A., Fujita, M., 2011 p. Glyoxalase system and reactive oxygen species detoxification system in plant abiotic stress response and tolerance: an intimate relationship. In: Shanker, A. (Ed.), Abiotic Stress in Plants—Mechanisms and Adaptations. InTech, pp. 235–266.

Huai, J., Zheng, J., Wang, G., 2009. Overexpression of a new Cys2/His2 zinc finger protein ZmZF1 from maize confers salt and drought tolerance in transgenic *Arabidopsis*. Plant Cell Tissue Org. 99, 117–124.

Hurkman, W.J., Lane, B.G., Tanaka, C.K., 1994. Nucleotide sequence of a transcript encoding a germin-like protein that is present in salt-stressed barley (*Hordeum vulgare* L.) roots. Plant Physiol. 104, 803–804.

Huynh, M.L., Russrll, P., Walsh, B., 2009. Tryptic digestion of in-gel proteins for mass spectrometry analysis. Method Mol. Biol. 519, 507–513.

Janas, K.M., Ciupińska, E., Posmyk, M.M., 2009. Melatonin applied by hydropriming, as phytobiostimulator improving corn (*Zea mays* L.) seedlings growth at abiotic stresses conditions. In: Li, S., Wang, Y., Cao, F., Huang, P., Zhang, Y. (Eds.), Progress in Environmental Science and Technology, Vol II A. Science Press USA Inc., pp. 383–388.

Janas, K.M., Posmyk, M.M., 2013. Melatonin, an underestimated natural substance with great potential for agricultural application. Acta Physiol. Plant 35, 3285–3292.

Karan, R., Subudhi, P.K., 2012. Overexpression of a nascent polypeptide associated complex gene of *Spartina alterniflora* improves tolerance to salinity and drought in transgenic *Arabidopsis*. Biochem. Biophys. Res. Commun. 424, 747–752.

Ke, Y., Han, G., He, H., Li, J., 2009. Differential regulation of proteins and phosphoproteins in rice under drought stress. Biochem Biophys. Res. Commun. 379, 133–138.

Kraft, E., Stone, S.L., Ma, L., Su, N., Gao, Y., Lau, O., Deng, X.W., Callis, J., 2005. Genome analysis and functional characterization of the E2 and RING-type E3 ligase ubiquitination enzymes of *Arabidopsis*. Plant Physiol. 139, 1597–1611.

Lane, B.G., 2002. Oxalate, germins, and higher-plant pathogens. IUBMB 53, 67–75.

Lauring, B.H., Sakai, G.K., Wiedmann, M., 1995. Nascent polypeptide-associated complex protein prevents mistargeting of nascent chains to the endoplasmic reticulum. Proc. Natl. Acad. Sci. U. S. A. 92, 5411–5415.

Lobell, D.B., Schlenker, W., Costa-Roberts, J., 2011. Climate trends and global crop production since 1980. Science 333, 616–620.

Lou, Y., Baldwin, I.T., 2006. Silencing of a germin-like gene in *Nicotiana attenuata* improves performance of native herbivores. Plant Physiol. 140, 1126–1136.

Lyzenga, W.J., Stone, S.L., 2012. Abiotic stress tolerance mediated by protein ubiquitination. J. Exp. Bot. 63, 599–616.

Manchester, L.C., Tan, D.X., Reiter, R.J., Park, W., Monis, K., Qi, W., 2000. High levels of melatonin in the seeds of edible plants—possible function in germ tissue protection. Life Sci. 67, 3023–3029.

Murata, S., Minami, Y., Minami, M., Chiba, T., Tanaka, K., 2001. CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO Rep. 2, 1133–1138.

Panasenko, O.O., David, F.P.A., Collart, M.A., 2009. Ribosome association and stability of the nascent polypeptide-associated complex is dependent upon its own ubiquitination. Genetics 181, 447–460.

Park, S., Lee, D.E., Jang, H., Byeon, Y., Kim, Y.S., Back, K., 2013. Melatonin rich transgenic rice plants exhibit resistance to herbicide-induced oxidative stress. J. Pineal Res. 54, 258–263.

Pla, M., Gomez, M., Goday, A., Pages, M., 1991. Regulation of the abscisic acid-responsive gene rab28 in maize viviparous mutants. MGG 230, 394–400.

Posmyk, M.M., Kuran, H., Marciniak, K., Janas, K.M., 2008. Pre-sowing seed treatment with melatonin protects red cabbage seedlings against toxic copper ion concentrations. J. Pineal Res. 45, 24–31.

Posmyk, M.M., Bałabusta, M., Janas, K.M., 2009a. Melatonin applied by osmopriming, as phytobiostimulator improving cucumber (*Cucumis sativus* L) seedlings growth at abiotic stresses conditions. In: Li, S., Wang, Y., Cao, F., Huang, P., Zhang, Y. (Eds.), Progress in Environmental Science and Technology, Vol II A. Science Press USA Inc., pp. 362–369.

Posmyk, M.M., Bałabusta, M., Wieczorek, M., Sliwinska, E., Janas, K.M., 2009b. Melatonin applied to cucumber (*Cucumis sativus* L.) seeds improves germination during chilling stress. J. Pineal Res. 46, 214–223.

Shi, H., Chan, Z., 2014. The cysteine2/histidine2-type transcription factor ZInc finger of *Arabidopsis thaliana* 6-activated C-repeat-binding factoR pathway is essential for melatonin-mediated freezing stress resistance in *Arabidopsis*. J. Pineal Res. 57, 185–191.

Shi, H., Qian, Y., Tan, D.X., Reiter, R.J., He, C., 2015. Melatonin induces the transcripts of CBF/DREB1s and their involvement in both abiotic and biotic stresses in *Arabidopsis*. J. Pineal Res. 59, 334–342.

Simon, M.I., Strathmann, M.P., Gautam, N., 1993. Diversity of G proteins in signal transduction. Science 252, 802–808.

Stirnimann, C.U., Petsalaki, E., Russell, R.B., Müller, C.W., 2010. WD40 proteins propel cellular networks. Trends Biochem. Sci. 35, 565–574.

Stone, S.L., 2014. The role of ubiquitin and the 26S proteasome in plant abiotic stress signaling. Front Plant Sci. 5, 1–10.

Tan, D.X., Manchester, L.C., Di Mascio, P., Martinez, G.R., Prado, F.M., Reiter, R.J., 2007. Novel rhythms of *N*-1-acetyl-*N*-2-formyl-5-methoxykynuramine and its precursor melatonin in water hyacinth: importance for phytoremediation. FASEB J. 21, 1724–1729.

Terrón, M.P., Marcgena, J., Shadi, F., Harvey, S., Lea, R.W., Rodriguez, A.B., 2001. Melatonin: an antioxidant at physiological concentrations. J. Pineal Res. 31, 95–96.

Thompson EW Lane BG, 1980. Relation of protein synthesis in imbibing wheat embryos to the cell-free translational capacities of bulk mRNA from dry and imbibing embryos. J. Biol. Chem. 255, 5965–5970.

Tnani, H., López, I., Jouenne, T., Vicient, C.M., 2012. Quantitative subproteomic analysis of germinating related changes in the scutellum oil bodies of *Zea mays*. Plant Sci. 191, 1–7.

Tolleter, D., Hincha, D.K., Macherel, D.A., 2010. Mitochondrial late embryogenesis abundant protein stabilizes model membranes in the dry state. Biochim. Biophys. Acta 1798, 1926–1933.

Wu, X., Liu, H., Wang, W., Chen, S., Hu, X., Li, C., 2011. Proteomic analysis of seed viability in maize. Acta Physiol. Plant 33, 181–191.

Zhang, N., Sun, Q., Zhang, H., Cao, Y., Weeda, S., Ren, S., Guo, Y.D., 2015. Roles of melatonin in abiotic stress resistance in plants. J. Exp. Bot. 66, 647–656.

Zhang, N., Zhao, B., Zhang, H.-J., Weeda, S., Yang Ch Yang, Z.-C., Ren, S., 2013. Guo Y-D. Melatonin promotes water-stress tolerance, lateral root formation, and seed germination in cucumber (*Cucumis sativus* L.). J. Pineal Res. 54, 15–23.

Zhang, Y., Yang, C., Li, Y., Zheng, N., Chen, H., Zhao, Q., Gao, T., Guo, H., Xie, Q., 2007. SDIR1 is a RING finger E3 ligase that positively regulates stress-responsive abscisic acid signaling in *Arabidopsis*. Plant Cell. 19, 1912–1929.

Zimmermann, G., Baumlein, H., Mock, H.P., Himmelbach, A., Schweizer, P., 2006. The multigene family encoding germin-like proteins of barley: regulation and function in Basal host resistance. Plant Physiol 142, 181–192.

Oświadczenia współautorów publikacji **Kołodziejczyk I., Dzitko K., Szewczyk R., Posmyk M.M. (2016)** Exogenous melatonin improves corn (*Zea mays* L.) embryo proteome in seeds subjected to chilling stress. *Journal of Plant Physiology* 193: 77-56 DOI:10.1016/j.jplph.2016.01.012 – wchodzącej w skład rozprawy doktorskiej:

| Imię i nazwisko | Szacunkowy udział [%] | Opis działań | Podpis |
|---|---|---|---|
| mgr Izabela Kołodziejczyk | 70 | Przygotowanie nasion – wykonanie hydrokondycjonowania (H, HMel50, HMel500). Hodowla materiału roślinnego w ustalonych warunkach eksperymentu. Ekstrakcja, oczyszczanie i zagęszczanie białek z osi zarodkowych. Separacja i wizualizacja białek techniką 2-D. Analizy i typowanie spotów za pomocą oprogramowania PDQuest. Przygotowanie materiału do analiz LC-MS/MS – izolacja i trawienie białek z żeli. Pozyskanie danych z odpowiednich baz – analiza porównawcza przy użyciu narzędzi MASKOT, NCBI i DELTA-BLAST. Opracowanie, analiza i interpretacja wyników. Przygotowanie manuskryptu. | Kołodziejczyk Izabela |
| dr hab. Katarzyna Dzitko, prof. UŁ | 5 | Konsultacja metodyczna dotycząca rozdziałów 2D. | Dzitko |
| dr Rafał Szewczyk | 10 | Wykonanie analiz LC–MS/MS z powierzonego materiału. Instruktaż w pozyskiwaniu danych z MASCOT, NCBI i przy użyciu algorytmu DELTA-BLAST. | |
| prof. dr hab. Małgorzata M. Posmyk | 15 | Współtwórca koncepcji pracy. Pomoc metodyczna na różnych etapach analiz. Obliczenia statystyczne. Pomoc w interpretacji wyników i redakcji manuskryptu. | |
| Dane biblio-metryczne z dn. 19.05.2021 r. wg. *Web.Sci.*: **IF 3.121**$^{2016}$, **3.615**$^{V}$; **30** cytowań. (**100**$^{A}$ pkt-y MEiN) | | | |

*Załącznik 5*

International Journal of
*Molecular Sciences*

MDPI

*Article*

# Melatonin application modifies antioxidant defense and induces endoreplication in maize seeds exposed to chilling stress

**Izabela Kołodziejczyk [1,*], Andrzej Kaźmierczak [2] and Małgorzata M. Posmyk [1]**

[1] Department of Plant Ecophisiology, Faculty of Biology and Environmental Protection, University of Lodz, 90237 Lodz, Poland; izabela.kolodziejczyk@biol.uni.lodz.pl (I.K.), malgorzata.posmyk@biol.uni.lodz.pl (M.M.P.)
[2] Department of Cytophysiology, Faculty of Biology and Environmental Protection, University of Lodz, 90237 Lodz, Poland; andrzej.kazmierczak@biol.uni.lodz.pl (A.K.)
* Correspondence: izabela.kolodziejczyk@biol.uni.lodz.pl; Tel.: +48-42-635-44-22

**Abstract:** The work concerns biostimulating properties of melatonin (MEL) applied to maize seeds by hydroconditioning. For the tests at the physiological, biochemical and cytological level, control, non-treated seeds (NT), hydroconditioned with pure water (H) and with aqueous MEL solutions at 50 and 500 μM concentrations (HMel 50 and HMel 500, respectively) were used. It was demonstrated that the application of exogenous MEL to seeds improved their germination under suboptimal temperatures and stimulated the growth of embryonic axes under chilling stress conditions (5 °C) and after stress removal, during regeneration. The antioxidant activity of MEL was confirmed by low level of protein oxidative damage and smaller quantity of lipid oxidation products in embryonic axes of HMel 50 and HMel 500 seeds exposed to chilling temperature 5 °C for 14 days. The activity of basic antioxidant and detoxifying enzymes was also analyzed. The stimulatory effects of MEL on SOD, peroxidases: APX and GSH-PX and on GST, a detoxifying enzyme were demonstrated. It was also showed, for the first time, that MEL induced defense strategies against stress at the cytological level, inducing endoreplication in embryonic axes cells partially even in the seeds germinating under optimal conditions (preventive action), but very intensively in those germinating under chilling stress conditions (intervention action), and after stress removal, to improve regeneration.

**Keywords:** antioxidant enzymes; biostimulation; chilling stress; corn seeds; endoreplication; melatonin; seed priming; *Zea mays*

**Citation:** Kołodziejczyk, I.; Kaźmierczak, A.; Posmyk, M.M. Melatonin application modifies antioxidant defense and induces endoreplication in corn maize exposed to chilling stress. *Int. J. Mol. Sci.* **2021**, *22*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Izabela Kołodziejczyk

Received: date
Accepted: date
Published: date



## 1. Introduction

The majority of plant cultivation in spite of various agrotechnical procedures is carried out in suboptimal conditions for plants, but also the changing climate exposes them to new stresses. Unfortunately, excessive agriculture chemicalization, application of fertilizers and synthetic plant protection products, is unfavorable for the environment. Hence the necessity of search for natural (biodegradable) factors, that through induction of internal defense strategies, might help plants overcome or adapt to stress [1,2].

One of the most important cultivated plants in the world is corn (*Zea mays* L.). The plant is an important part of human diet, and in the face of prevalence of food allergies, the absence of gluten in corn grains increases their value on the cereal market [3,4]. Moreover, green parts of corn are the fodder for farm animals, and biodegradable, fully ecological materials are produced from its fibbers. Because this plant comes from South America, the climate requirements for its growth are not entirely achieved in temperate climate zone, even for adapted varieties. Particularly temperature conditions for germination are suboptimal and limiting [5], therefore every low-cost improvement of sprouting might bring measurable increase in harvests and economic benefits.

The quality of seed material is a primary and basic criterion determining good yields. Thus, finding the effective techniques to improve sowing material seems to be a crucial point to combat potential stressors [6,7]. Conditioning is a highly effective method for improvement of seeds vigor [8]. However, to succeed, the water application method must be properly adjusted to a type (species) of seeds. Selection of the method should involve physiology, biochemistry and anatomy of seeds, the value of initial seed moisture and experimentally determined final value. The final moisture value in one that initiates the germination process, but does not allow the growth of the embryo and promotes secondary desiccation - the necessary step in any type of conditioning [9-12]. This technique can be combined with other supporting factors such as aeration, light-irradiation, and temperature-stratification. What is most important, various soluble bioactive substances (growth regulators, fungicides, biostymulators) can be introduced with water into the seeds [2,13,14].

Many scientists as well as breeders consider development of new natural biostimulators as the most promising method for ecological crops production that favors healthy food and protection of the environment. Our research experience suggest, that melatonin (MEL) has a significant phytobiostimulation potential, however, the effect of its, usually positive activity, is resultant of many factors [2,15]. The research on MEL role in plants has been significantly intensified recently [16,17]. Scientists are interested both in (1) the role, regulation and endogenous phytomelatonin synthesis [17-20] and (2) effects of exogenous MEL application in plants, most often throughout supplementation of substrates, or spraying [21-24]. The presented work is follows the second trend, yet in our research MEL is applied into seeds *via* conditioning.

MEL antioxidative activity is well documented [25-28]. It detoxifies a variety of free radicals and reactive oxygen species (ROS) including hydroxyl radical ($OH^*$), peroxynitrite anion ($ONOO^-$), singlet oxygen ($^1O_2^*$), and nitric oxide (NO) [29]. One of the most appealing properties of this molecule, which distinguishes it from most antioxidants, is the fact that its metabolites also have the ability to scavenge ROS and reactive nitrogen species (RNS) [30,31]. Melatonin generates free radical scavenging cascade providing continuous cell defense, which makes this molecule, even at low concentrations, highly effective in protecting organisms from oxidative stress [32,33]. There are also scientific reports suggesting influence of MEL on the activity of antioxidant enzymes [25-27,34] and their genes expression [35-37] – thereby on multidirectional effect on red-ox status in plant tissues. Direct and indirect antioxidant properties of MEL are particularly important in the face of environmental stress. Various stressors inhibit plant growth via different mechanisms, however all of them cause increase in ROS concentration. Over-production of these reactive compounds that is unfollowed by their neutralization disturbs redox homeostasis and induces oxidative stress, which is a well-known secondary effect of all biotic and abiotic stresses. Additionally to the fact that antioxidant activity is very desirable in stressed plant cells, it is worth mentioning that ROS can also be formed naturally as products of biochemical reactions involved in various physiological cycles (e.g., water-water cycle, cell-wall biosynthesis, and detoxification processes) and they also play important role in signaling pathways in plants. Thus they should not be completely eliminated but successfully restrained.

Oxidative stress blockade has a protective effect on biological membranes [38]. Owing to this property, MEL indirectly delays plant aging and limits damage in chloroplasts and mitochondria [39-41].

The purpose of the presented work is to document positive effects of MEL as biostimulator, supplemented during corn seed hydropriming, and study how exogenous melatonin can affect redox status of cells and modify antioxidant enzyme activity in embryonic axes isolated from corn seeds pretreated with MEL. Comparison was made between differently primed seeds variants: hydroconditioned with water (H), hydroconditioned with melatonin: 50 and 500 μM water solutions (HMel 50, HMel 500 respectively) and control – untreated ones (NT). There were three experimental procedures of

germination for each seed variant: (i) optimal conditions at 25 °C (C), (ii) chilling stress at 5 °C (S), (iii) chilling stress with subsequent recovery at 25 °C (R).

Furthermore, our previous proteomic analyzes [14,42] have shown significant quantitative and qualitative differences between proteomes from embryos treated with MEL, untreated and water hydroconditioned. The results of these tests formed the grounds for the assumption, that enlarging the protein pool in MEL treated seed embryos can be a result of DNA amount multiplication. Therefore, in the presented work we investigate the correlation between the exogenous MEL application and the polyploidization in plants. We dealt with the issue of multiple replications of genetic material – endocycles.

We are the first to check whether MEL actually triggers the switchover of cell cycles to endocycles in maize. Based on densitometric measurements of the embryonic basal zones, after reference of fluorescence intensity of the genetic material in individual nuclei, to the basic amount of DNA, histograms of genetic material distribution were created and a percentage of polyploid nuclei were obtained.

## 2. Results

### *2.1. Hydropriming condition determination*

There are two ways to control the moisture of the seeds during imbibition, (1) by limiting the quantity of water added directly to the seeds or (2) by controlling the time of seed soaking.

In the former case, attention should be paid to homogeneous access of seeds to a given water amount calculated for their specific portion (weight) [43]. Limited amount of seeds (small portions) could be used in this technique and hydropriming should be carried out in rotating containers. Unfortunately, angular seeds, as dry corn, can be injured during rotations - as it was observed in our preliminary experiments. In the latter case, when unlimited quantity of water is used, the time of soaking to obtain experimentally established final seed moisture content should be precisely determined. In this case kinetics of imbibition at optimal temperature 25 °C was determined (Figure 1). It was shown that 3 h of corn seed soaking at 25 °C was sufficient to obtain 36.77% ±2 of moisture content, thus hydropriming was done as follows: portions of 200 g of corn seeds were placed in plastic containers with 200 ml of oxygenated, distilled water or MEL water solutions (depending on the experimental variant) and incubated for 3 h at 25 °C. Than the seeds were air-dried at room temperature for the subsequent 3 days (to let them return to the initial water content) and used in tests.

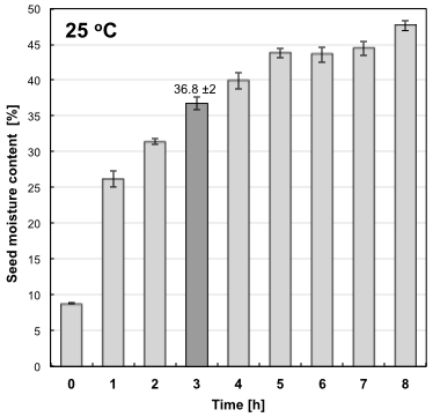



**Figure 1.** Kinetics of maize seed imbibition with distilled water at 25 °C.

### *2.2. MEL and IAA content in seeds*

HPLC-EC analyzes of NT, H, HMel 50 and HMel 500 seed extracts confirmed the efficacy of hydropriming supplemented with melatonin. The content of MEL increased 30 times in HMel 50 seeds and 600 times in HMel 500 ones compared to the untreated seeds. Hydropriming with water did not affect the amount of endogenous MEL – it remained at a level similar to that observed in NT seeds (Table 1).

**Table 1.** Effect of different seed hydropriming treatments on melatonin and IAA content in maize seeds. Seeds were hydroconditioned with water (H) or with melatonin water solution at concentrations: 50 and 500 μM (HMel 50 and HMel 500 respectively). Control seeds were not primed (NT). The results were expressed as means of 9-12 measurements ±SEM. One-way ANOVA and Duncan's post-hoc test were performed. The lowercase letters next to the values show the statistical significance at the specified p.

| Seed variants | Melatonin [ng gFW$^{-1}$] | IAA [μg gFW$^{-1}$] |
|---|---|---|
| NT | 35 ± 5 *a* | 2.63 ± 0.21 *b* |
| H | 31 ± 2 *a* | 3.50 ± 0.4 *c* |
| HMel 50 | 1 058 ± 58 *b* | 2.07 ± 0.13 *b* |
| HMel 500 | 20 830 ± 902 *c* | 1.13 ± 0.14 *a* |

[1] Melatonin – ANOVA results: Seed Variant (NT, H, HMel 50, HMel 500) $F_{(3; 25)}$=675.0, $p$<0.000001.
IAA – ANOVA results: Seed Variant (NT, H, HMel 50, HMel 500) $F_{(3; 34)}$=13.96, $p$<0.000005.

Hydropriming with water provoked a statistically significant increase in the level of IAA in the seeds, while MEL applied during such treatment caused the inverse effect, i.e. a decrease in IAA content proportional to the concentration of MEL used during hydropriming (Table 1).

### *2.3. Seed germination*

Maize seeds used in the experiments were of good quality – in optimal conditions (25 °C) they achieved 96% of germination on the second day and 100% on the fifth day of the experiment (Figure 2A). Therefore, under these conditions positive effects of pre-sowing treatments cannot be seen, as all variants of the seeds germinated well, the exception was HMel 500, with the surprising 13% germinated only (Figure 2A).

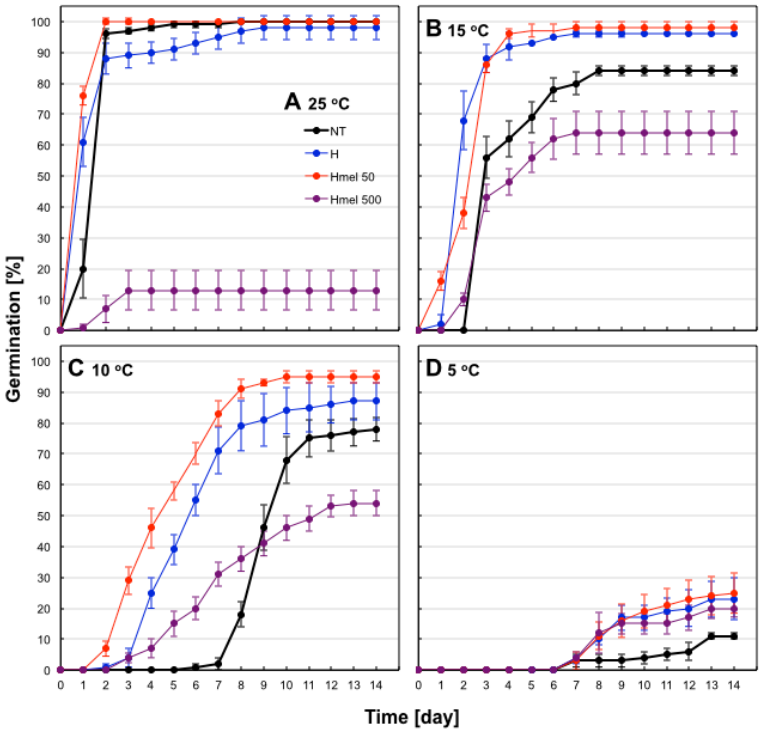



**Figure 2.** Effect of different maize seed hydropriming treatments on seed germination at optimal, 25 °C (A) and chilling: 15 (B), 10 (C) and 5 °C (D) temperatures. The seeds were hydroprimed with water (H – blue line) or with MEL water solution at concentrations 50 μM (HMel 50 – red line) and 500 μM (HMel 500 – purple line). Non-treated seeds (NT – black line) were not primed. Vertical bars represent ±SEM (*n*=4).

Lowering the temperature to 15 °C resulted in a slowdown of germination rate, the lag phase prolongation to 2 days and germination decrease to 84% in the control seeds - NT (Figure 2B). Germinability of H and HMel 50 seeds decreased very slightly - to 96% and 98%, respectively. Interestingly, at the suboptimal temperature, HMel 500 seeds began to germinate better then at optimal temperature, reaching a maximum of 64% on day 7 (Figure 2B)

Further lowering the temperature to 10 °C prolonged the lag phase of NT seeds to 6-7 days and reduced their germination to 77% (Figure 2C). In contrast, the conditioned seeds germinated from the 3rd day (H and HMel 500) and even the 2ed in the case of HMel 50. The obtained results were 87%, 95% and 54% for H, HMel 50 and HMel 500, respectively (Figure 2C).

Only 5 °C temperature significantly blocked germination of maize. Single germinations appeared on days 6-7 in all seed variants, and by the 14th day of the experiment only 11%, 23%, 25% and 20% of NT, H, HMel 50 and HMel 500 seeds germinated, respectively (Figure 2D). At 5 °C, a marked slowdown in the metabolic activity of seeds was observed, a small number could be considered germinated but in the case of every conditioned seed variant it was at least twice more compared to the NT ones. At these chilling conditions almost no seedlings developed. On the basis of the presented germination tests, 5 °C was chosen as a chilling stress temperature for maize seeds and suitable experimental scheme of seed germination and seedling growth conditions was created (see M&M – C, S, R).

### *2.4. Embryonic axes growth*

During germination at optimal temperature, 25 °C, the embryonic axes of HMel 50 seeds developed the best. In the other variants of seeds (NT, H, Mel500), their axes

growth was similar at 25 °C, but more than half weaker (56-58%) in comparison with HMel 50 (Figure 3).

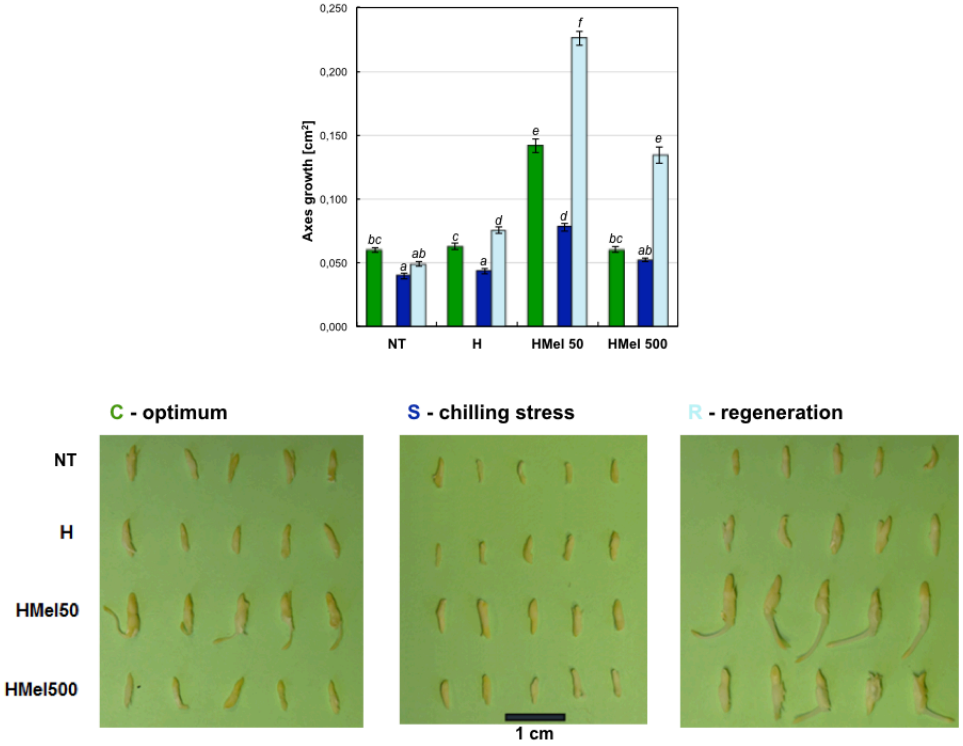



**Figure 3.** Effect of different maize seed hydropriming treatments on growth of the axes isolated from the non-treated seeds (NT), and those from hydroprimed with water (H) and with MEL water solutions in concentrations: 50 μM (HMel 50) and 500 μM (HMel 500). The seeds were imbibed/germinated in water at 5 ºC in darkness for 14 days (S [■] – chilling stress), and subsequently regenerated at 25 ºC for 1 day (R [ ] – regeneration). Growth was also estimated in axes isolated from the seeds germinated for 24 h under optimal conditions 25 ºC (C [■] – optimum, control conditions). The results presented on graph are expressed as means of 20-40 measurements ±SEM. Two-way ANOVA and Duncan's post-hoc tests were performed. The lowercase letters next to the values show statistical significance at the specified *p*. The results are also presented as a photo panel.

GROWTH – ANOVA results: Seed Variant $_{(NT, H, HMel\ 50, HMel\ 500)}$ $F_{(3; 348)}$=368.9, $p$<0.000001; Conditions $_{(C, S, R)}$ $F_{(2; 348)}$=267.4, $p$<0.000001; interaction Seed Variant x Conditions $F_{(6; 348)}$=67.2, $p$<0.000001.

The chilling stress, 5 °C, significantly inhibited the growth of embryonic axes in NT, H and HMel 500, while in the case of HMel 50 the limiting effect was attenuated and 49, 45 and 33% better results were observed, respectively. Interestingly, the chilled HMel 50 axes were also statistically significantly higher (by 20-23%) compared to the axes of NT, H and HMel 500 seeds developing under optimal conditions of 25 °C. However, when comparing the embryonic axes of HMel 50 seeds growing in optimal conditions with chilled ones, it is clearly visible that in this seed variant the growth was also significantly (45%) limited by cold stress (Figure 3).

Seed conditioning treatments improved their regenerative potential after the chilling stress. Even hydroconditioning with pure water improved growth after the stress removal by 36% compared to the non-conditioned (NT) variant, while in the seeds hydroconditioned with MEL solutions the effects were spectacular. The embryonic axes in the regenerated HMel 50 and HMel 500 seeds were 4.6 and 2.7 times larger, respectively, compared to the analogous NT ones (Figure 3).

### *2.5. Oxidative injury estimation*

Under optimal germination conditions, the level of compounds formed as a result of lipid peroxidation (TBARS) remained at a low and similar level in embryonic axes from all seed variants (slightly higher in NT) (Figure 4). In the seed embryos incubated for 14 days at chilling 5 °C (S), the degree of lipid oxidation increased significantly 2.7 and 2.5 times in NT and H, respectively and about 2 times in HMel 50 and HMel 500, comparing with the results obtained under optimal conditions. Although the trend of changes in TBARS level was the same in all seed variants under the given incubation conditions (C, S, R) - so the interaction of Seed Variant x Conditions was not statistically significant - the absolute values always showed statistically significant weaker lipid peroxidation in melatonin pretreated seeds comparing with NT ones - especially under chilling stress (Figure 4). After transferring the germinating seeds from chilling back to the optimal temperature (R), the downward trend of TBARS was observed in all seeds but in NT this decrease was not statistically significant. After regeneration (R), once again the weakest of oxidative damage of lipids was observed in embryonic axes isolated from the seeds pretreated with melatonin (HMel 50 and HMel 500) (Figure 4).

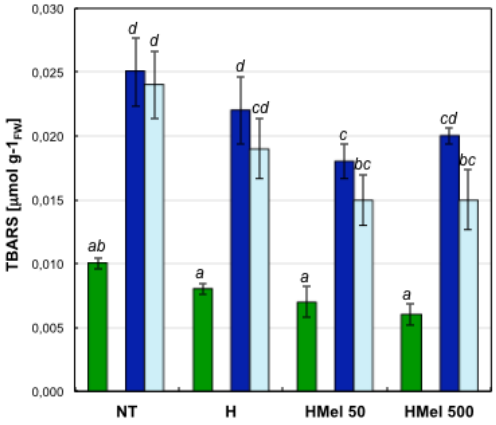



**Figure 4.** Effect of different maize seed hydropriming treatments on TBARS accumulation in the axes isolated from the non-treated seeds (NT), and those from hydroprimed with water (H) and with melatonin water solutions in concentrations: 50 µM (HMel 50) and 500 µM (HMel 500). The seeds were imbibed/germinated in water at 5 ºC in darkness for 14 days (S [■] – chilling stress), and subsequently regenerated at 25 ºC for 1 day (R [□] – regeneration). TBARS were also estimated in axes isolated from the seeds germinated for 24 h under optimal conditions 25 ºC (C [■] – optimum, control conditions). The results are expressed as means of 6-9 measurements ±SEM. Two-way ANOVA and Duncan's *post-hoc* tests were performed. The lowercase letters next to the values show statistical significance at the specified *p*.

TBARS – ANOVA results: Seed Variant $_{(NT, H, HMel\ 50, HMel\ 500)}$ $F_{(3; 84)}$=6.57, $p$<0.000001; Conditions $_{(C, S, R)}$ $F_{(2; 84)}$=44.07, $p$<0.000001; interaction Seed Variant x Conditions $F_{(6; 84)}$=0.80, $p$=0.57 no statistical significance.

The degree of protein carbonylation in embryonic axes of the seeds germinating under optimal conditions was also similar (Figure 5). The 14-day chilling stress (S) strongly increased the concentration of carbonyl groups in the proteins extracted from NT seed embryos and to a lesser extent from H seed embryos (Figure 5). Unfortunately, the upward trend of this parameter was maintained in the above seed variants even after the stress relieve during regeneration (R) (Figure 5). In contrast, in embryos of HMel 50 seeds an insignificant increase in protein carbonylation was observed under chilling stress and after its removal and in the case of HMel 500 even decrease in this parameter (Figure 5). In both cases, the actual reduction of oxidative modifications / damages to proteins probably thanks to MEL applied to the seeds can be postulated.

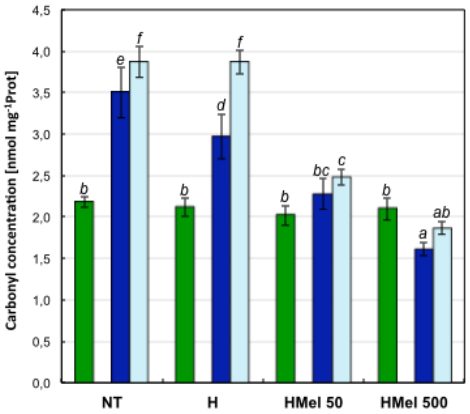



**Figure 5.** Effect of different maize seed hydropriming treatments on protein oxidation expressed as carbonyl group concentration in the axes isolated from non-treated seeds (NT), and those from hydroprimed with water (H) and with melatonin water solutions in concentrations: 50 μM (HMel 50) and 500 μM (HMel 500). The seeds were imbibed/germinated in water at 5 ºC in darkness for 14 days (S [■] – chilling stress), and subsequently regenerated at 25 ºC for 1 day (R [□] – regeneration). Proteins and its oxidation were also estimated in axes isolated from the seeds germinated for 24 h under optimal conditions 25 ºC (C [■] – optimum, control conditions). The results are expressed as means of 6-9 measurements ±SEM. Two-way ANOVA and Duncan's post-hoc tests were performed. The lowercase letters next to the values show statistical significance at the specified *p*.

Carbonyls – ANOVA results: Seed Variant (NT, H, HMel 50, HMel 500) $F_{(3; 84)}$=35.9, $p$<0.000001; Conditions (C, S, R) $F_{(2; 84)}$=25.0, $p$<0.000001; interaction Seed Variant x Conditions $F_{(6; 84)}$=7.83, $p$<0.000001.

### *2.6. Antioxidant and detoxifying enzymes activities*

Smaller oxidative damages observed were correlated with a better functioning of antioxidant enzyme system. SOD already under optimal conditions exhibited higher activity in the HMel 50 and in particular HMel 500 seed embryos (Figure 6A). Chilling significantly reduced SOD activity in the embryonic axes of NT and H seeds, and it did not increase even after their regeneration at optimal conditions. In the axes of seeds treated with MEL, cold also decreased SOD activity but only by 1/3 of the control value. Moreover, both in stress and after regeneration, it remained about twice as high as in NT and H seeds (Figure 6A).

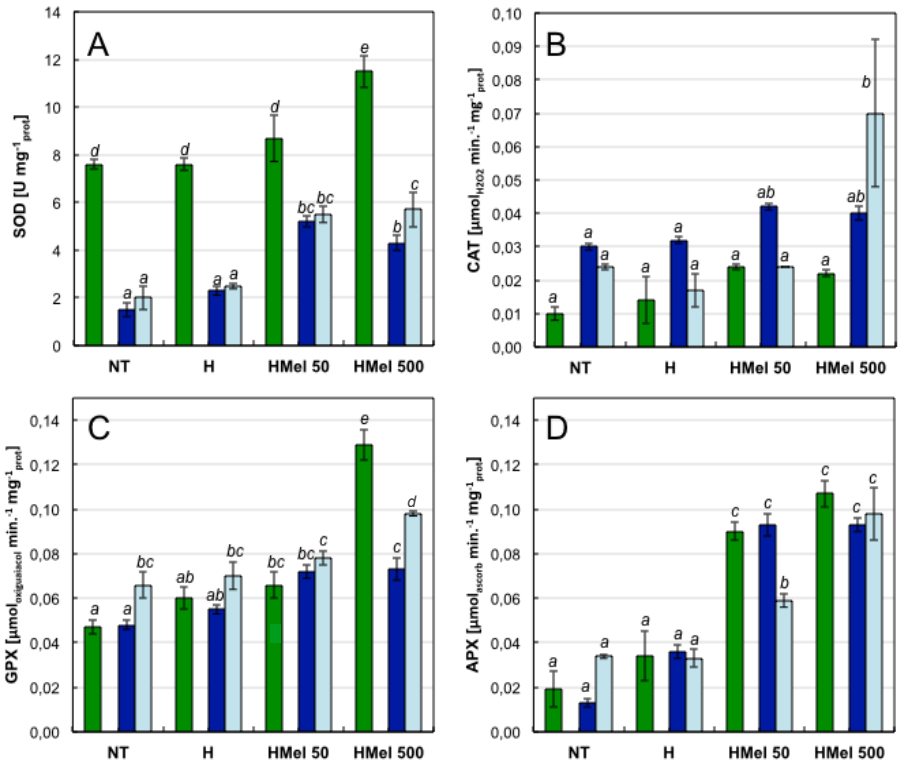



**Figure 6.** Effect of different maize seed hydropriming treatments on the antioxidant enzymes activities: SOD (A), CAT (B), GPX (C) and APX (D) in the axes isolated from non- treated seeds (NT), and those from hydroprimed with water (H) and with melatonin water solutions in concentrations: 50 μM (HMel 50) and 500 μM (HMel 500). The seeds were imbibed/germinated in water at 5 ºC in darkness for 14 days (S [■] – chilling stress), and subsequently regenerated at 25 ºC for 1 day (R [ ] – regeneration). Enzyme activities were also estimated in axes isolated from the seeds germinated for 24 h under optimal conditions 25 ºC (C [■] – optimum, control conditions). The results are expressed as means of 6-9 measurements ±SEM. Two-way ANOVA and Duncan's post-hoc tests were performed. The lowercase letters next to the values show statistical significance at the specified *p*.

SOD – ANOVA results: Seed Variant (NT, H, HMel 50, HMel 500) $F_{(3; 79)}$=39.69, $p$<0.000001; Conditions (C, S, R) $F_{(2; 79)}$=167.53, $p$<0.000001; interaction Seed Variant x Conditions $F_{(6;79)}$=2.96 $p$<0.01.

CAT – ANOVA results: Seed Variant (NT, H, HMel 50, HMel 500) $F_{(3; 88)}$=4.33, $p$<0 005; Conditions (C, S, R) $F_{(2; 88)}$=4.32, $p$<0.01; interaction Seed Variant x Conditions $F_{(6; 88)}$=2.09, $p$=0.062 no statistical significance.

GPX – ANOVA results: Seed Variant (NT, H, HMel 50, HMel 500) $F_{(3; 82)}$=75.27, $p$<0,000001; Conditions (C, S, R) $F_{(2; 82)}$=16.69, $p$<0.000001; interaction Seed Variant x Conditions $F_{(6; 82)}$=14.79, $p$<0.000001.

APX – ANOVA results: Seed Variant (NT, H, HMel 50, HMel 500) $F_{(3; 77)}$=77.32, $p$<0.000001; Conditions (C, S, R) $F_{(2; 77)}$=0.71, $p$=0,049 no statistical significance; interaction Seed Variant x Conditions $F_{(6; 77)}$=3.13, $p$<0.005.

There were no significant changes in CAT activity, which was low in embryonic axes isolated from all seed variants. Its activity increased after the chilling stress relief in the case of HMel 500, however, SEM of the values obtained was high, which suggests significant heterogeneity of the material tested with regard to this parameter (Figure 6B).

It seems that in the studied plant material, peroxidases played the main role in eliminating oxidative stress. At optimal conditions 25 ºC their activity slightly increased in H and HMel 50 seeds in comparison to NT. The highest activity was observed in HMel 500 ones (Figure 6C). It is important that, peroxidases were not chilling-sensitive and they kept their activity during the stress. In the non-specific peroxidase test (GPX), in the embryonic axes of NT and H seeds the highest enzymatic activity of all the antioxidant enzymes tested at 5 ºC, was observed (Figure 6C). After stress removal during

regeneration (R) their activity increased in all seed variants. Moreover this activity was higher in HMel 50 and HMel 500 as compared to the NT variant. The greatest GPX activity was observed in axes of HMel 500 seeds under optimal conditions: C and R (Figure 6C).

Detailed peroxidase tests showed, that MEL applied into the seeds significantly stimulated the APX activity in the axes of HMel 50 and HMel 500 variants under all experimental conditions C, S and R (Figure 6D). In contrast, the activity of APX in NT and H was small, as was the activity of GSH-PX (Figure 7A). Under optimal conditions (C), MEL application significantly stimulated the activity of GSH-PX – 10 fold in the case of HMel 50 and 12 fold in the case of HMel 500 comparing with NT. While in the case of GST activity this effect was much less intensive, the increases were 2- and 1.5-fold for HMel 50 and HMel 500, respectively comparing to NT and H (Figure 7C). Only GSSG activity was similar in all experimental seed variants germinated at optimal conditions (C) (Figure 7B). The studied glutathione-associated enzymes appear to be sensitive to 5 °C chilling (Figure 7ABC) - the exception was GSSG-R in the HMel 50 and HMel 500 seed axes, where its activity under stress conditions (S) doubled compared to NT and H. High activity of GSSG-R in the melatonin treated seeds was also maintained after stress, during regeneration (R) (Figure 7B). The activity of the detoxifying GST enzyme was also significantly greater in the embryonic axes from melatonin treated seeds (HMel 50 and HMel 500) during recovery after chilling stress (R) (Figure 7C).

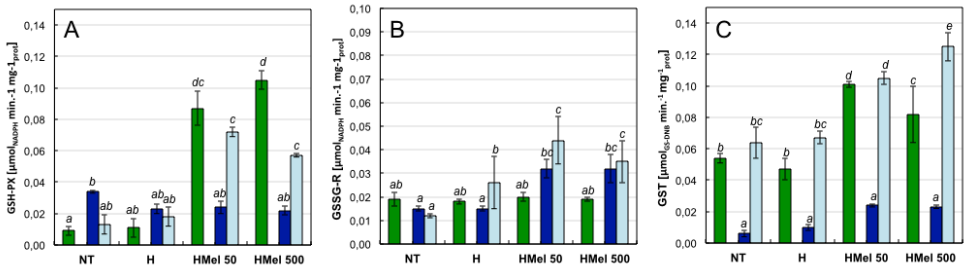



**Figure 7.** Effect of different corn seed hydropriming treatments on the glutathione-dependent detoxification enzymes activities: GSH-PX (A), GSSG-R (B), and GST (C) in the axes isolated from non treated seeds (NT), and those from hydroprimed with water (H) and with melatonin water solutions in concentrations: 50 μM (HMel 50) and 500 μM (HMel 500). The seeds were imbibed/germinated in water at 5 ºC in darkness for 14 days (S [■] – chilling stress), and subsequently regenerated at 25 ºC for 1 day (R [ ] – regeneration). Enzyme activities were also estimated in axes isolated from the seeds germinated for 24 h under optimal conditions 25 ºC (C [■] – optimum, control conditions). The results are expressed as means of 6-9 measurements ±SEM. Two-way ANOVA and Duncan's *post-hoc* tests were performed. The lowercase letters next to the values show statistical significance at the specified *p*.

GSH-PX – ANOVA results: Seed Variant (NT, H, HMel 50, HMel 500) $F_{(3; 81)}$=33.27, $p$<0.000001; Conditions (C, S, R) $F_{(2; 81)}$=13.06, $p$<0.000001; interaction Seed Variant x Conditions $F_{(6; 81)}$=14.62, $p$<0.000001.

GSSG-R – ANOVA results: Seed Variant (NT, H, HMel 50, HMel 500) $F_{(3; 118)}$=8.52, $p$<0.0001; Conditions (C, S, R) $F_{(2; 118)}$=5.68, $p$<0.005; interaction Seed Variant x Conditions $F_{(6; 118)}$=2.60, $p$<0.01.

GST – ANOVA results: Seed Variant (NT, H, HMel 50, HMel 500) $F_{(3; 77)}$=24.61, $p$<0.000001; Conditions (C, S, R) $F_{(3; 77)}$=24.61, $p$<0.000001; interaction Seed Variant x Conditions $F_{(6; 77)}$=3.79, $p$<0.005.

### *2.7. Analysis of cell distribution in the determined cell cycle phases*

The target of our work was also to determine whether there was a connection between pre-sowing MEL application into maize seeds and endoreplication stimulation in the embryos basic zone. The microcytphotometric measurements of nuclei stained DAPI fluorochrome (Figure 8A-J) and their digital transformation (Figure 8A'-J') allowed us to observe different karyokinesis phases (Figure 8A-F), endocycle appearance (Figure 8G-H) as well as nuclei fragmentation (Figure 8I-J).

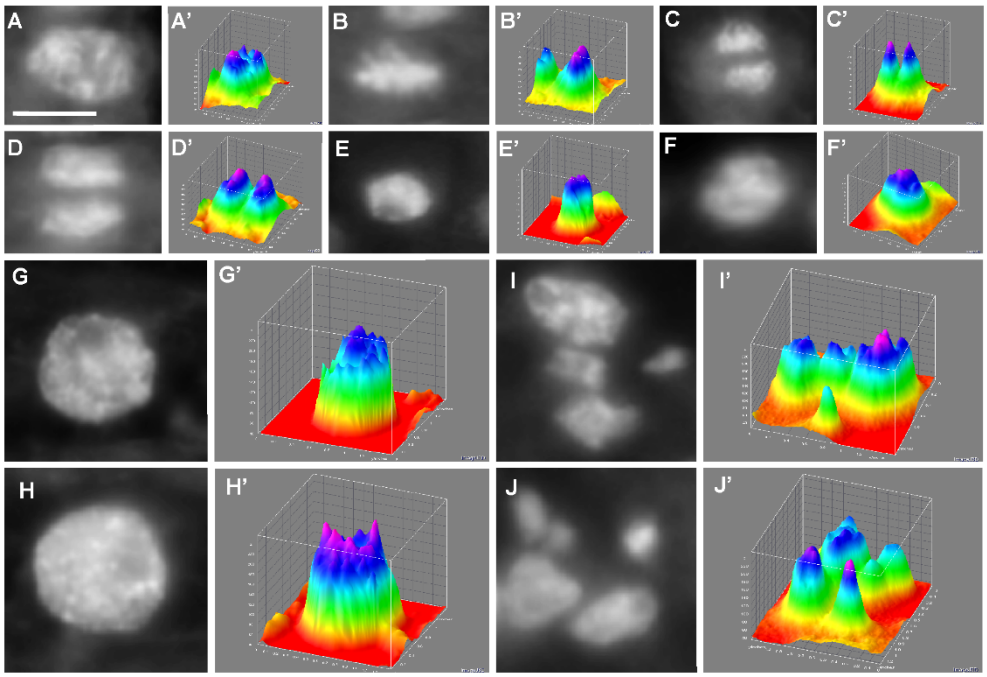



**Figure 8.** Micrographs (A-J) and their digital transformations (A′-J′), of nuclei from *Zea mays* axes basic zone cells fixed in Carnoy's and stained with DAPI showing: prophase (A, A′), metaphase (B, B′), anaphase (C, C′) and telophase (D, D′), nucleus during under the G1 (E, E′) and under the G2 (F, F′) phases and nuclei in endocycle, i.e. endo S with 6C (G, G') and with 8C DNA (H, H′) as well as nuclei fragmentation (I, I′ and J, J′). Scale bar is 10 μm (A). The photos and digital transformations represent cells from NT seeds germinated at optimal temperature (Control): A, A′, B, B′, C, C′, D, D′ and F, F′; from hydroconditioned H seeds germinated at optimal temperature (Control): E, E′; from HMel 50 seeds after regeneration phase (R): G, G′ and H, H; from hydroconditioned H seeds after regeneration phase (R): I, I′ and J, J′.

The densitometric measurements of DAPI fluorochrome allowed us to conclude that exogenous MEL stimulates endocycle appearance in embryonic axes even during germination at optimal temperature 25 °C (C) (Figure 9). The histograms on Figure 9 are characteristic of undifferentiated tissues, where proliferation takes place and the slow, constant embryo growth ensues. It was important, that in the embryos isolated from MEL-treated seeds the polyploid nuclei occurred - especially in HMel 50.

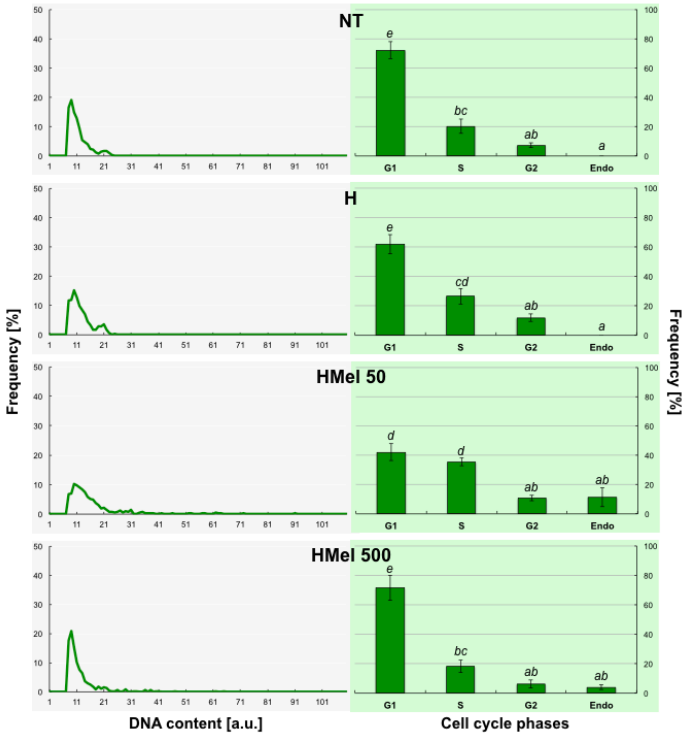



**Figure 9.** Effect of different maize seed hydropriming treatments on DNA content (line charts) and relative number of cells in G1, G2, S and > 4C phases/Endo (bar graphs) in the axes isolated from non treated seeds (NT), and those from hydroprimed with water (H) and with melatonin water solutions in concentrations: 50 μM (HMel 50) and 500 μM (HMel 500). The seeds were imbibed/germinated for 24 h under optimal conditions 25 ºC (C [■] – optimum, control conditions). The results of cell cycle phase distribution are expressed as means of 5 analyses ±SEM. Two-way ANOVA and Duncan's post-hoc tests were performed. The lowercase letters next to the values show statistical significance at the specified *p*.

Cell cycle phases distribution – ANOVA results: Seed Variant $_{(NT, H, HMel\ 50, HMel\ 500)}$ $F_{(3; 64)}$=0, $p$=1; Cell Phase $_{(G1, S, G2, Endo)}$ $F_{(3; 64)}$=136.07, $p$<0.0000001; interaction Seed Variant x Cell Phase $F_{(9; 64)}$=4.85, $p$<0.0001.

The histograms received for seeds germinated at 5 ºC (Figure 10) revealed that the proliferation process was disturbed by chilling (S). In NT and H seed variants, cells were mostly stopped in G1 phase, and relatively few cells were observed in other cycle phases. As we supposed, the largest number of endoreplicating cells (about 40% cell nuclei in HMel 50 and 20% in HMel 500) appeared in MEL-treated seeds.

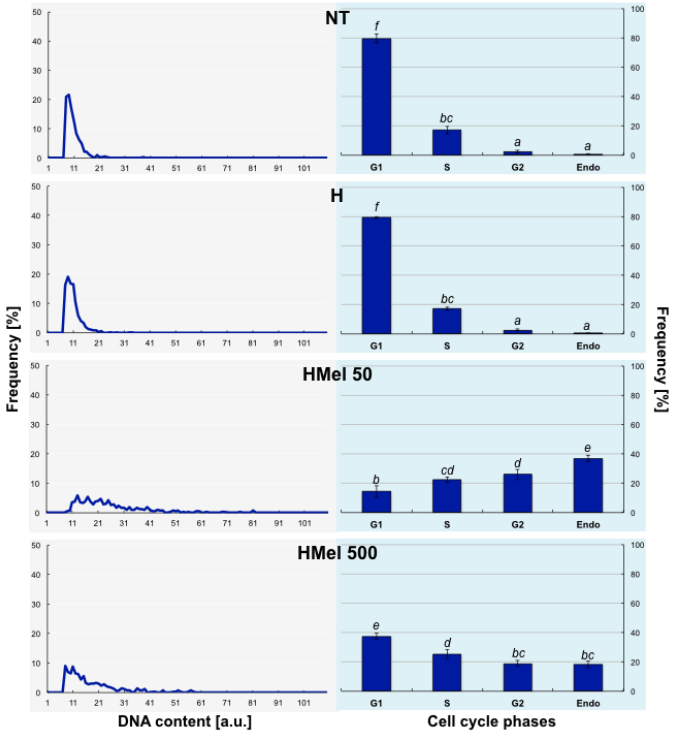



**Figure 10.** Effect of different maize seed hydropriming treatments on DNA content (line charts) and relative number of cells in G1, G2, S and > 4C phases/Endo (bar graphs) in the axes isolated from non treated seeds (NT), and those from hydroprimed with water (H) and with melatonin water solutions in concentrations: 50 μM (HMel 50) and 500 μM (HMel 500). The seeds were imbibed/germinated in water at 5 ºC in darkness for 14 days (S [■] – chilling stress). The results of cell cycle phase distribution are expressed as means of 5 analyses ±SEM. Two-way ANOVA and Duncan's post-hoc tests were performed. The small lowercase next to the values show statistical significance at the specified *p*.

Cell cycle phases distribution – ANOVA results: Seed Variant $_{(NT, H, HMel\ 50, HMel\ 500)}$ $F_{(3; 64)}$=0, $p$=1; Cell Phase $_{(G1, S, G2, Endo)}$ $F_{(3; 64)}$=291.08, $p$<0.0000001; interaction Seed Variant x Cell Phase $F_{(9; 64)}$=102.89, $p$<0.0000001.

The measurements made after stress in recovery period (R) (Figure 11) showed that in the case of embryonic axes isolated from NT and H seeds the state observed earlier at chilling stress became prevailing – almost 100% of nuclei were in G1 phase - they did not return to proliferation and nuclei fragmentations were also observed (Figure 8I-J). These seeds were in very bad condition. On the other hand, again we detected a lot of polyploid nuclei – endo-S phase (6C) and tetraploid nuclei (Figure 8G and H, respectively) – in MEL-treated grains, particularly in HMel 50 (about 40%), that resulted in the fast growth and regeneration of seedlings (Figure 3).

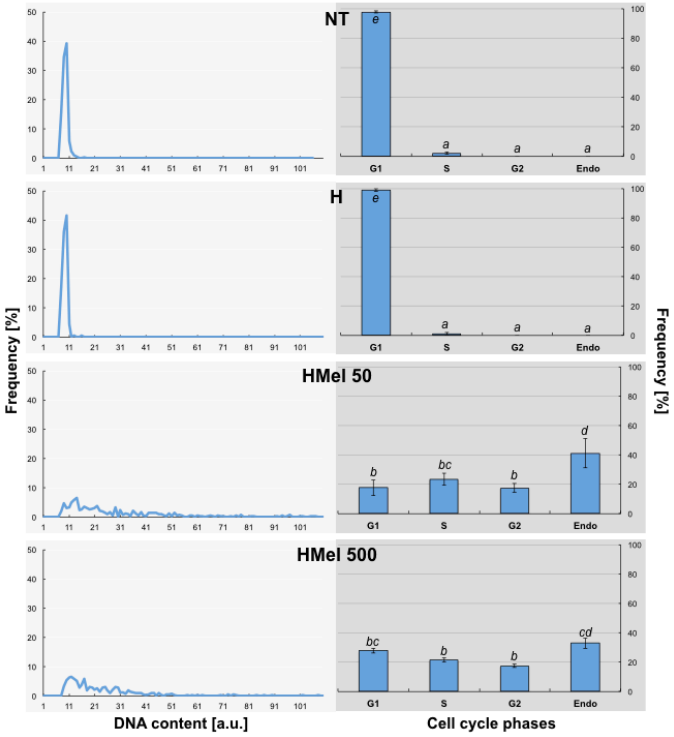



**Figure 10.** Effect of different maize seed hydropriming treatments on DNA content (line charts) and relative number of cells in G1, G2, S and > 4C phases/Endo (bar graphs) in the axes isolated from non treated seeds (NT), and those from hydroprimed with water (H) and with melatonin water solutions in concentrations: 50 μM (HMel 50) and 500 μM (HMel 500). The seeds were imbibed/germinated in water at 5 ºC in darkness for 14 days and subsequently regenerated at 25 ºC for 1 day (R [■] – regeneration). The results of cell cycle phase distribution are expressed as means of 5 analyses ±SEM. Two-way ANOVA and Duncan's *post-hoc* tests were performed. The lowercase letters next to the values show statistical significance at the specified *p*.

Cell cycle phases distribution – ANOVA results: Seed Variant (NT, H, HMel 50, HMel 500) $F_{(3; 64)}=0$, $p=1$; Cell Phase (G1, S, G2, Endo) $F_{(3; 64)}=207.15$, $p<0.0000001$; interaction Seed Variant x Cell Phase $F_{(9; 64)}=78.56$, $p<0.0000001$.

## 3. Discussion

Maize (*Zea mays* L.) is cereal greatly important for people and animals as nutritional ingredient as well as it may be processed into other industrial biodegradable products [4]. In order to be efficient a cultivation of maize in temperate climate zone has to be constantly strengthened by various treatments. We suggest, that it is possible with application of proper methods of pre-sowing seed conditioning.

There are three main types of seeds conditioning: (1) hydroconditioning (2) osmoconditioning and (3) matriconditioning [8-10,13]. All this methods are based on controlled seed hydration leading to limited imbibition, without embryonic axes growth initiation. Final MC has to prevent radical growth and emergence, that allows for seed secondary desiccation to initial MC and than their safety storage until sowing. Final MC, although typical for the seed species, should always be determined experimentally for given seed lot.

First, and the simplest priming - water soaking – is a rapid method (time measured in hours), wherein the water availability might be controlled by (i) its quantity given to the seeds (it has to be calculated for known mass of seeds) or (ii) the time of its accessibility (soaking period has to be chosen on the basis of earlier examined kinetics of the seed imbibition – as shown in Figure 1). During osmoconditioning water availability for seeds is limited by a low osmotic potential of the prepared osmotically active solution, which means, that seeds must be incubated in it longer (time measured in days). Simi-

larly, during matriconditioning low water potential (matrioptential) of specific solid carriers is used. All the conditioning techniques mentioned may be supplemented with an application of growth stimulators, protective components and other bioactive substances (so-call chemical priming).

The seeds with high starch content, including maize (it contains approximately 72% of starch) [4], endures osmopriming badly. Prolonged (measured in days) seed incubation in increased moisture causes losing ability to safe and proper secondary desiccation - so it disturbs the last stage of conditioning. It is probably related to the activation of alpha-amylases and irreversible mobilization of resources by decomposition of starch. In consequence, osmoconditioned maize seeds germinate worse unfortunately than the non-conditioned ones (results non shown). We documented that experimentally well-matched hydroconditioning technique applied to corn seeds provided satisfying results both in seed germination (Figure 2) and embryonic axes growth (Figure 3). The effects of seed priming was additionally enhanced by the use of a natural biostimulator – melatonin.

The positive effect of hydropriming, and especially hydropriming with MEL, is clearly visible in germination tests carried out under suboptimal temperature (Figure 2). Additionally we observed higher regenerative potential of maize embryos after chilling stress 5 °C (Figure 3). The surprising effect of a significant reduction in germination of HMel 500 seeds variant in optimal conditions of 25 °C can be explained by excessive reduction of ROS due to a very high concentration of MEL in these seeds (Table 1). Too low ROS level disturb phenomenon of the so-called 'oxidative window' necessary to sustain correct signal transduction during germination [44]. This effect is gradually eliminated at decreasing temperatures, where chilling stress replenishes the 'missing' ROS pool in this case.

The level of endogenous melatonin in plants varies depending on the species, organ, age and growth conditions [45,46]. The seeds seem to be exceptionally rich with it. The studied maize grains contained 30-35 ng endogenous MEL per gram fresh weight (Table 1) – that is an average result compared to the richest mustard seeds ($129^{-1}89$ ng $g^{-1}$ [47]) however, such a concentration is incomparably higher than that recorded in animal tissues. As it was found, that phytomelatonin is synthesized particularly vigorously in suboptimal conditions and melatonin-rich plant species showed higher capacity for stress tolerance [48-50] it seemed advisable to provide the seeds with this idoleamine preventively. For this purpose, the hydropriming technique was successfully applied (Table 1).

Melatonin is structurally similar to IAA, but the functions of these two molecules differ. MEL more intensely stimulates the growth of aboveground parts of plants [51], although similar to IAA, it also stimulates root development. All positive effects of MEL activity are especially visible during environmental stresses. In tomato (*Lycopersicon esculentum* Mill.) [52] and brown mustard (*Brassica juncea* L.) [53] treated with exogenous MEL (0.05, 0.01 μM), a slight, transient increase in IAA concentration was observed, but it decreased over the time [54]. In contrast, overproduction of MEL in *Arabidopsis thaliana* L. and tomato mutants [55] and treatment of plants with a higher concentration of exogenous MEL (above 0.5 μM) caused a significant decrease of IAA level. The effect of MEL on IAA seems to depend on the MEL quantity used, however, the method of its application and targeted plant organ are also important. In our experiments, a much higher concentrations of MEL were used than in studies discussed above. The application of 50 μM MEL to corn seeds did not change the IAA level, only 500 μM MEL reduced its amount to 43% of the control. However, it should be noted, that the hydropriming (seed treatment method) increased the level of IAA in seeds by about 35%, hence the negative correlation was visible only at a higher MEL concentration used. We have indirectly demonstrated similarly to Wang *et al.* (2016) [56], that MEL negatively regulates auxin concentration in seeds. However, since various studies show both positive and negative correlation between MEL and IAA, this problem should be considered

in a wider network of relationships modulating the expression of auxin-related transcription factors [57,58].

In the presented study, we indicated that MEL applied into the seeds modifies the activity of basic antioxidant and detoxifying enzymes, reducing secondary oxidative stress and chilling injuries.

Cold stress intensifies lipid and protein peroxidation. We have shown that embryonic axes from MEL treated seed variants cumulate significantly less TBARS (products of lipid oxidation) than NT ones, both during stress and after it has subsided (Figure 4). Moreover, the degree of protein carbonylation analysis showed that the seeds treated with MEL had no (HMel 500) or slight (HMel 50) increase in proteins oxidation comparing with its level at optimal temperature condition (Figure 5). Such a protection against chilling stress of protein-lipid membranes and the enzymes functioning in them is certainly the reason for the efficient regeneration and faster growth of the embryonic axes from MEL-treated seeds as documented in Figure 3. The results above are very consistent with those presented by Cao *et al*. (2019) [59], who conditioned wax maize seeds with MEL (50 and 100 μM) and then observed seedlings grown from them under constant chilling of 13 °C. Moreover, the reduction of ROS accumulation in rice seedlings exposed to 12 °C chilling was also observed after the application of MEL (20 and 100 μM) administered *via* seed soaking, seedling root immersing or leaves spraying [39]. The positive effects increased with the MEL concentration used. Similarly, spraying with 1 mM MEL solution of wheat seedlings [60] and maize seedlings [61] exposed to cold 2-5 °C was selected as significant inhibitor of oxidative damage, maintaining membrane functionality. Gao *et al*. (2018) [38] successfully applied 100 μM MEL during 28 days cold storage of peach fruit. The MEL-treated fruit had a lower level of lipid peroxidation - better membrane integrity than the untreated ones. Thus, the reduction of ROS, lower lipid peroxidation, and decrease in relative electrolyte leakage (a measure of membrane integrity) under the influence of MEL in many plants exposed to various environmental stresses are well documented [56,62-64]. It is certainly related to the MEL direct antioxidant properties as well as its influence on the antioxidant enzymes activity.

Our research showed that although SOD from the embryonic axes of maize seeds seems to be sensitive to 5 °C cold. Its activity decreased significantly at low temperature and did not regenerate, after the stress subsides, to the activity level achieved at 25 °C by plants incubated under optimal conditions all the time. However, despite that, under and after cold stress, SOD activity was 2-2.5 times higher in seeds pre-sowing treated with MEL than in NT and H ones. (Figure 6A). Especially intensely stimulated by MEL were peroxidases: of ascotrbate (APX - Figure 6D) and of glutathione (GSH-PX - Figure 7A), as well as glutathione S-transferase (GST - Figure 7C); and although the mentioned glutathione-dependent enzymes (GSH-PX and GST) appear to be also sensitive to the low temperature of 5 °C (under cold they performed poorly), thus after transferring the germinating seeds of HMel 50 and HMel 500 to 25 °C they regained remarkably efficient functionality quickly. While glutathione reductase (GSSG-R) in seeds hydroconditioned with MEL was twice as active both under cold stress and after its disappearance, compared to that in NT and H ones. This helps to maintain a favorable pool of reduced glutathione under stress and during regeneration after it has subsided, which is in line with the data published by Bałabusta *et al.* (2016) [65], that involved cucumber seeds osmoconditioned with MEL.

Cold is a specific stress - due to deceleration of biochemical reactions and the preservative effect of low temperatures, it is sometimes difficult to observe negative effects during its operation. That is why observations and analyses performed after plants have been transferred to optimal conditions are so important - cold damage is most clearly visible during the regeneration period at a higher temperature.

In a thematically similar work by Cao *et al.* (2019) [59] it was documented a clearly stimulating and directly proportional to the concentration used (MEL 50 and 100 μM), the effect of melatonin applied to wax maize seeds, on the activity of all tested antioxi-

dant enzymes, i.e. SOD, CAT, APX and non-specific peroxidases. However, it should be noted, that the chilling stress used in the experiments mentioned above was milder (13 °C) and short-lived (up to 5 days) and probably for this reason it did not strain the activity of cold-sensitive enzymes. Similarly, in the work of Han *et al.* (2017) [66] MEL of 20 and 100 μM, applied *via* various ways - into the seeds, roots or leaves of rice seedlings, stimulated the activity of SOD, CAT and non-specific peroxidases, especially at the higher concentration of MEL (100 μM) used. But also in this case, plants were exposed to milder stress of 12 °C for 6 days. Moreover the examined organs - the leaves of 12 and 14 days old seedlings - were different than our material (embryonic axes) discussed in this work. Most of the available publications describing the effect of exogenous MEL on the antioxidant enzymes activity concern the developing seedlings of various species i.g. of wheat [60], corn [61], rice [66] – plant material isolated from germinating seeds was analyzed rarely. The vast majority of works confirm that exogenous MEL applied to plants provoke a positive effects by intensifying (although in slightly various ways) the activity of antioxidant enzymes in face of cold / chilling stress, that in consequence reduces symptoms and damage generated by secondary oxidative stress.

Our latest results [14,42] indicated that MEL seed treatment expediently modified proteome of maize (*Z. mays* L.) embryo during seed germination. The majority of additional proteins were (i) energy metabolism enzymes,(ii) proteins involved in proteome plasticity via improving protein synthesis, folding, destination and storage, and – most importantly – (iii) defense, anti-stresses, and detoxifying proteins. This explains why seeds hydroconditioned with MEL and seedlings grown from them were stronger in comparison to the non-treated ones, and quickly and efficiently adapted to changing environmental conditions. They were a priori prepared to cope with potential harmful conditions. These results partially explain how melatonin acts in plant stress defense and why various plant species rich in MEL have shown higher capacity for stress tolerance [67].

Because it was observed not only qualitative but also quantitative increase in the protein pool of embryonic axes isolated from the seeds treated with MEL, the questions appeared: how is this possible and especially how is this possible during chilling stress when the metabolism is slowing down?

It is well known that in polyploid cells' more efficient and more productive metabolism is observed. Endocycles multiply the number of copies of the desired gene, maximizing mRNA availability and protein production. This phenomenon occurs both during optimal plant development and under harmful environmental conditions [68]. It has been shown that polyploid cells adapt better to environmental changes, including unfavorable ones [69,70]. Polyploidization is associated with the phenomenon of endoreplication - also known as the endocycle. This process is much less understood than the standard cell cycle. However, it has been noticed that plant mutants with suppression of auxin synthesis, transport and signaling show fast mitosis switching to the endocycle and achieve an increased level of polyploidy, which allows cells to grow faster [70]. These known facts, consistent with our results - of increased protein synthesis, lower IAA levels, and faster seedling growth due to MEL application into the maize seeds - prompted us to verify whether MEL can also induce endoreplication in growing maize embryonic axes.

Cells that have entered the endocycles quickly increase their size without losing the materials for building organelles, which would have to be duplicated before classic division, and their enlargement describes the caryoplasmic factor (the greater the content of genetic material in the nucleus, the greater the cell surface area). This positively influences the rapid growth of plant, and the duplication of genes allows the intensification of certain attributes such as flower color, scent, fruit flavor, but also the content of desirable compounds e.g. with antioxidant properties [68,69,71].

In the preparations from the basal zone of maize embryonic axes, we observed exceptionally large cells, and after staining with DAPI, the typical endocycle nuclei inside

624 them (Figure 8 GG'HH'). They were most often observed in the HMel 50 seed variant
625 and appeared during their incubation under chilling stress (Figure 10) and after it had
626 subsided - during regeneration phase (Figure 11). They were also observed, but less fre-
627 quently, during the incubation of these seeds in optimal conditions (Figure 9). It should
628 be remembered that at the same moments of the experiment the embryos HMel 50 grew
629 best (Figure 3) compared to the remaining seed variants Application of MEL at a con-
630 centration 10 times higher - 500 μM - also provoked endocycles but less effectively than
631 in the case of 50 μM MEL seed treatment. It seems that the MEL concentration used is
632 crucial for the occurrence of endocycles, although the sensitivity to it may also vary de-
633 pending on the organ examined, and certainly the plant species. Wang *et al.* (2017) [72]
634 showed that high concentration of MEL (1000 μM) suppresses the leaf growth in *Ara-*
635 *bidopsis thaliana* L. by reduction of cell size and cell number. Their further comprehensive
636 analyses suggested that MEL might regulate the leaf growth by inhibiting cell prolifera-
637 tion and endoreduplication too. However, mentioned above observations were carried
638 out under optimal for plant growth conditions. Our results are the first that indicate the
639 relationship of MEL application effect with the occurrence of endocycles during, and af-
640 ter chilling stress - as a strategy for increasing plant survival.
641 It is known that endoreplication occurs as a natural way for plants to compensate
642 evolutionary losses in small genome and an evolutionary strategy to obtain more of the
643 final products that plants need and synthesize in the different environmental conditions
644 they have to face [73-76]. Hence it would be useful to have a tool that would allow to
645 induce and control such a strategy to improve the quality and quantity of crops. In our
646 opinion, MEL is a good candidate for this, but further research is necessary in this topic.

647 **4. Materials and Methods**

648 *4.1. Plant Material*

649 Maize seeds (*Zea mays* L. var. Ambrozja) were delivered by TORSEED (Torun, Po-
650 land). They were stored in the dark, in dry conditions at room temperature, in tightly
651 closed containers before the experiments started.

652 *4.2. Hydropriming*

653 The maize seeds were hydroprimed according to the methods described by Taylor
654 *et al.* (1998) [43] with our modifications. Seed water content (MC – moisture content) was
655 calculated as the percentage of fresh weight basis (1):
656

$$MC = \frac{FW - DW}{FW} \cdot 100\% \quad (1)$$

657
658 First, the seeds were weighed (FW) and then after 3 days of their incubation at 108
659 ºC their dry mass (DW) was also weighed. Their initial moisture content was 8.84%
660 ±0.23.
661 To establish crucial water content in imbibed seeds before radical protrusion, the
662 seeds were imbibed in different PEG-8000 solutions giving an osmotic potential between
663 –1 and –2 MPa [77]. After 7 days of imbibition with PEG – 1.5 MPa the corn seeds did
664 not germinate (there were no radical protrusion observed – the seeds imbibed, but em-
665 bryos were not grown), so of 38.01% ±1.12 MC was chosen as sufficient for corn seed
666 metabolic activity to occur while preventing radical growth and emergence.
667 The technique and hydropriming conditions were determined experimentally as: 3
668 h of seed soaking at 25 °C (see Results). Oxygenated, distilled water (H) or MEL water
669 solutions 50 (HMel 50) and 500 (HMel 500) μM were used depending on the experi-
670 mental variant.

Than the seeds were re-dried at room temperature for the subsequent 3 days (time sufficient for returning to the initial water content) and than used in physiological, biochemical and cytological tests.

### *4.3. Germination test*

The seeds placed on 9-cm diameter Petri dishes with 2 layers of Wht 2 filter paper wetted with distilled water were germinated in darkness at 5-25 °C temperature range. The germination test was performed on samples of 100 seeds: 25 seeds per dish, 4 replicates. A seed was scored as germinated when its coat was broken and a radicle was visible. Germination counts were made daily up to 14 days. The results presented are the means of the values obtained in 4 replicates ± SEM.

### *4.4. Seed germination and seedling growth conditions*

The seeds (variants: NT, H, HMel 50, HMel 500) placed on 9-cm diameter Petri dishes (30 seeds per dish) with two layers of Whatman 2 filter paper (Whatman International Ltd. Maidstone, UK) wetted with distilled water were imbibed/germinated: (i) at optimal temperature 25 $^{o}$C for 24 h (C - control), (ii) at chilling stress temperature 5 $^{o}$C for 14 days (S - chilling stress), (iii) at 5 $^{o}$C for 14 days and then subsequently at 25 $^{o}$C for 24 h (R, recovery / regeneration after stress). Corn embryonic axes isolated from seeds/young seedlings were used for growth visualization and for biochemical and cytological tests.

The growth of embryonic axes was estimated by analyzing their photographs using ImageJ, an image-processing program designed for scientific multidimensional images, which converts pixels to cm$^2$ based on the indexed scale. The results are the means of 20-40 measurements ±SEM.

### *4.5. Melatonin and indole-3-acetic acid determination*

The concentrations of MEL and indole-3-acetic acid (IAA) were determined using high-performance liquid chromatography with electrochemical detection (HPLC-EC).

#### 4.5.1. Extraction procedure

The seeds (1 g) were homogenized with 5 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA. The homogenate was incubated for 30 min. at room temperature in darkness with minimal shaking, in order to ensure complete extraction of indoles. Then, it was centrifuged at 15 000 g for 20 min at 8 °C. Initial purification consisted in two-step solvent-partitioning using ethyl acetate (2 x 10 ml). First at the initial phosphate buffer pH 8.0 and second after pH changing to 3.0. The two organic phases (~10 ml each) were evaporated together under vacuum. The dry residue was re-dissolved in 1 ml of mobile phase, filtered through Supelco ISO-Disc filters (PTEF-4-2.4 mm x 0.2 μm), and frozen at -70 °C until HPLC-EC analysis. When analyzed, 10 μl of each filtrate was injected into the HPLC-EC system.

#### 4.5.2. HPLC-EC analysis

The HPLC system consisted of a quaternary gradient delivery pump Model 1050 (Agilent Technology), a sample injector Model 7125 (Rheodyne, Berkeley) and an analytical column ZORBAX SB-C18 3.0 x 250 mm, particle size 5 μm (Agilent Technology) protected by an analytical guard column ZORBAX SBC18 4.6 x 12.5 mm, (particle size 5 μm Agilent Technology). The electrochemical detector model HP 1049 A (Agilent Technology), with glassy carbon working electrode, was used at a voltage setting of +0.80V vs. an Ag/AgCl reference electrode. The detector response was plotted and measured using a Chromstation ver. A.08.03 (Agilent Technology).

719 The mobile phase contained 0.15 M sodium dihydrogen phosphate, 0.1 mM EDTA,
720 0.5 mM sodium octanesulphonic acid, 20% (v/v) methanol and 5 mM lithium chloride.
721 The mobile phase was adjusted to pH 3.4 with phosphoric acid, filtered through What-
722 man nylon membrane filter (45 μm) and degassed with helium. The column temperature
723 of 32 °C and flow rate of 0.8 ml $min^{-1}$ were used.
724 The concentrations of all compounds in each sample were calculated from the inte-
725 grated chromatographic peak height on the basis of standards, IAA and MEL, calibra-
726 tion curves, and expressed as ng MEL or μg IAA of $g^{-1}$ fresh weight (FW) of tissues. The
727 results are the means of 6-9 measurements ±SEM.

728 *4.6. TBARS test*

729 Lipid peroxidation was evaluated from the resultant products. Thiobarbituric acid
730 reactive substances (TBARS): aldehydes, mainly malondialdehyde (MDA) and en-
731 doperoxides were determined according to the methods described by Hodges *et al.*
732 (1999) [78]. MDA, routinely used as an indicator of lipid peroxidation, was extracted
733 with 1% (w/v) trichloroacetic acid (TCA). The reaction with 0.5% (w/v) thiobarbituric
734 acid (TBA) in 20% (w/v) TCA was conducted at 95 °C for 30 min. After the samples were
735 chilled in ice, specific absorbance was measured at 532 nm and non-specific absorbance
736 at 600 nm. The results were calculated using a molar absorption coefficient of 155 000 $M^{-1}$
737 $cm^{-1}$ and expressed as MDA μmol $g^{-1}$ of fresh weight (FW) and they are the means of 9
738 measurements ±SEM.

739 *4.7. OxiProt test*

740 Protein oxidation was estimated in terms of total carbonyl group content in modi-
741 fied proteins [79]. Protein carbonyl groups reacted with 2,4-dinitrophenyl hydrazine
742 (DNPH) to yield the corresponding 2,4-dinitrophenyl hydrazones, spectrophotometri-
743 cally measured at 370 nm.
744 Samples, 250 mg of fresh tissue (axes isolated from germinating seeds), were ho-
745 mogenized in extraction buffer (3 ml) consisting of 100 mM phosphate buffer (pH 7.5), 1
746 mM EDTA and 2 mM dithiothreitol (DTT) as follows. Each homogenate was centrifuged
747 at 20 000 g for 30 min. at 4 ºC. Then the supernatant fraction was filtered through Mira-
748 cloth and used for assays. The protein content was determined according to the Brad-
749 ford (1976) [80] method using bovine serum albumin (BSA) as a standard. To 0.5 ml of
750 extract (aliquot of 500-800 μg protein), 0.5 ml of 10 mM DNPH in 2 M HCl was added
751 and vortexed every 10 min for 1 h at room temperature. Corresponding protein blanks
752 were prepared by adding 0.5 ml of 2 M HCl instead of DNPH. After incubation, 0.5 ml
753 of 30% (w/v) TCA was added for protein precipitation, the samples were vortexed, then
754 placed on ice for 15 min. Following centrifugation at 10 000 g for 15 min at 4 ºC, the su-
755 pernatant was discarded and the pellets were subjected to extensive washing (three
756 times) with 1 ml of mixture ethanol:ethyl acetate (1:1; v/v) to remove any unreacted
757 DNPH. Finally, the pellets were solubilized in 1 ml of 6 M guanidine hydrochloride in
758 5% (w/v) phosphoric acid at 37 ºC (water bath) for 45 min with shaking.
759 Carbonyl contents were determined from the absorbance at 370 nm using a molar
760 absorption coefficient of 22 000 $M^{-1}$ $cm^{-1}$ [81]. The results are the means of 9 measure-
761 ments ±SEM.

762 *4.8. Enzyme extraction procedure*

763 One gram of fresh weigh of axes was ground in a mortar and homogenized with 0.5
764 g polyvinylpyrrolidone (PVP) in 5 ml of 0.1 M phosphate buffer (pH 7.5) containing 2.5
765 mM DTT, 1 mM EDTA, 1.25 mM PEG-4000 and 1 mM phenylmethylsulfonyl fluoride
766 (PMSF). The homogenate was centrifuged at 15 000 rpm for 30 min in 4 ºC. The resulting
767 supernatant was filtered through Miracloth, desalted on a PD10 column (Pharmacia,

Uppsala, Sweden) and used for the enzyme assays. All steps of the extraction procedure were carried out at 4 °C.

*4.9. Enzyme activity tests*

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured according to Giannopolitis and Ries (1977) [82]. The reaction mixture contained 2 μM riboflavine, 13 mM methionine, 0.1 mM EDTA, 70 μM nitrotetrazolium blue chloride (NBT) in 0.1 M phosphate buffer (pH 7.5), and 100 μl of the enzyme extract in the final volume of 3 ml. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT. Glass test tubes containing the mixture were illuminated with a fluorescent lamp at 25 °C (Philips MLL 5000 W, Eindoven, the Netherlands). Identical tubes, which were incubated in darkness served as blanks. After illumination for 60 min, absorbance was measured at 560 nm. One unit of SOD was defined as the enzyme activity, which inhibited the photoreduction of NBT to blue formazan by 50 %. SOD activity was expressed as the enzyme unit per milligram of protein [U $mgprot^{-1}$].

Catalase (CAT, EC 1.11.1.6.) activity was measured according to Clairbone (1985) [83]. The enzyme assay mixture contained 18 mM $H_2O_2$ in 0.1 M phosphate buffer (pH 7.0) and 100 μl of the enzyme extract in the total volume of 2 ml. CAT activity at 25 °C was estimated by the decrease in $H_2O_2$ determined from the absorbance at 240 nm monitored for 100 s. The molar absorption coefficient of 40 000 $M^{-1}$ $cm^{-1}$ [84] was used for calculations. The results were expressed as micromoles of $H_2O_2$ decomposed during 1 minute per 1 milligram of protein [μmol $min^{-1}$ $mgprot^{-1}$].

Non-specific peroxidases (GPX) activity was measured according to Scebba *et al*. (2001) [85]. The reaction mixture contained 2.25 mM guaiacol, 11 mM $H_2O_2$ in 0.1 M phosphate buffer (pH 6.0), and 100 μl of the enzyme extract in the total volume of 2 ml. GPX activity was assayed at 25 °C by following the increase in oxidized guaiacol determined from the absorbance at 470 nm monitored for 300 s. The molar absorption coefficient of 26 600 $M^{-1}$ $cm^{-1}$ [84] was used for calculations. The results were expressed as micromoles of guaiacol oxidized during 1 minute per 1 miligram of proteins [μmol $min^{-1}$ $mgprot^{-1}$].

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed according to Kato and Shimizu (1987) [84]. The reaction mixture contained 1.2 mM EDTA, 35 mM $H_2O_2$, 15 mM L-ascorbic acid, 0.1 M phosphate buffer (pH 7.0) and 200 μl of the examined extract. APX activity at 25 °C was estimated by the decrease in ascorbate determined from the absorbance at 290 nm monitored for 300 s. The molar absorption coefficient of 2 800 $M^{-1}$ $cm^{-1}$ [84] was used for calculations. The enzyme activity was expressed as μM L-ascorbic acid decreased in 1 min per 1 mg of proteins [μmol vit C $min^{-1}$ mg $prot^{-1}$].

Glutathione peroxidase (GSH-PX, EC 1.11.1.9) activity was determined according to Nagalakshmi and Prasad (2001) [86]. The assay mixture contained 1 M NaCl, 10 mM EDTA, 10 mM reduced glutathione (GSH), 8 mM NADPH, 25 mM $H_2O_2$, GSSG-R (200 U $ml^{-1}$), 0.1 M phosphate buffer (pH 8.0) and 100 μl of the examined extract. GSH-PX activity at 30 °C was assayed by the decrease in NADPH determined from the absorbance at 340 nm monitored for 10 min. The molar absorption coefficient of 6 200 $M^{-1}$ $cm^{-1}$ [87] was used for calculations. The enzyme activity was expressed as μmol of NADPH decreased during 1 min per 1 mg of proteins [μmol NADPH $min^{-1}$ mg $prot^{-1}$].

Glutathione reductase (GSSG-R, EC 1.6.4.2) activity was determined according to Esterbauer and Grill (1978) [88]. The assay mixture contained 0.5 mM NADPH, 10 mM glutathione disulfide (GSSG), 6.25 mM $MgCl_2$ in 0.1 M phosphate buffer (pH 7.5), and 100 μl of the enzyme extract in the total volume of 400 μl. GSSG-R activity at 30 °C was estimated by the decrease in NADPH determined from the absorbance at 340 nm monitored for 20-30 min. The molar absorption coefficient of 6 200 $M^{-1}$ $cm^{-1}$ [87] was used for calculations. GSSG-R activity was expressed as micromoles of NADPH decreased during 1 minute per 1 miligram of proteins [μmol $min^{-1}$ $mgprot^{-1}$].

Glutathione S-transferase (GST; EC 2.5.1.18) activity was determined according to Habig, *et al.* (1974) [89] and Nagalakshmi and Prasad (2001) [86]. The assay mixture contained 10 mM GSH, 100 mM 1-chloro-2,4-dinitrobenzene (CDNB), 0.1 M phosphate buffer (pH 8.0) and 200 μl of the examined extract. Activity of GST was measured at 30 $^{o}$C by following the increase in GS-DNB at 340 nm during 300 sec. The molar absorption coefficient of 9 600 $M^{-1}$ $cm^{-1}$ [89] was used for calculations The enzyme activity was expressed as μmol of GS-DNB composed during 1 min per 1 mg of proteins [μmol GS-DNB $min^{-1}$ mg $prot^{-1}$].

SOD, CAT, GPX, APX, GSH-PX, GSSG-R and GST activities of each extract were measured 3 times, and the results presented correspond to the means ± SEM of the values obtained with 3 different extracts (n = 9). Protein content of the extracts was determined according to the method of Bradford (1976) [80].

### *4.10. DAPI staining*

The DAPI staining was accomplished according to Byczkowska *et al.* (2013) [90] and Kaźmierczak (2010) [91] methods. The isolated embryos were fixed in cold Carnoy's containing 96% ethanol and glacial acetic acid, in proportion 3:1 for 1 h, and then washed with ethanol series of dilutions, finally with distilled water. Then they were cut to slides with cryostat (CM1950, Leica) and stained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) by 5-min pretreatment with 0.2 M citric acid and 0.1% Tween. The dyeing solution contained 3.2 mM DAPI (2 μg $ml^{-1}$) in mixture of 0.1 M Na2HPO4 and 0.2 M citric acid in 9:1 ratio. After 5 min staining, the axes were washed with the buffer mixture, and subsequently analyzed using a fluorescence microscope (Optiphot-2, Nikon, Japan) with UV2A filter, and photographed using ACT-1 digital camera (Precoptic, Poland). The microphotographs were used for microcytophotometric measurements and digital transformations using ImageJ software.

### *4.11. Statistical analyses*

The results represent the average values ± standard error of the mean (±SEM). The data were analysed using STATISTICA v.10.0_MR1_PL [StatSoft] software. The two-way analysis of variance (ANOVA) and then the post-hoc Duncan multiple range test were carried out to find the significant differences which were determined at $p<0.01$ in each experiment.

**Supplementary Materials:** there are no supplementary materials.

**Author Contributions:** Conceptualization – K.I. and P.M.M.; methodology – K.I., K.A. and P.M.M.; validation – K.I., K.A. and M.M.P.; investigation – K.I., K.A.; writing—original draft preparation – K.I.; writing—review and editing – P.M.M..; visualization – K.I., K.A..; supervision – P.M.M..; funding acquisition – P.M.M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Acknowledgments:** The microscopic slides with plant material were prepared in the Laboratory of Microscopic Imaging and Specialized Biological Techniques, Faculty of Biology and Environmental Protection, University of Lodz.

**Conflicts of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Abbreviations**

$^{1}O_{2}^{*}$ – singlet oxygen
ANOVA – statistical analysis of variance
APX – ascorbate peroxidase

BSA – bovine serum albumin
C – optimal, control conditions
CAT - catalase
CDNB – 1-chloro-2,4-dinitrobenzene, citric acid
DAPI – diamidine-2'-phenylindole dihydrochloride
DTT – dithiothreitol
DNA - deoxyribonucleic acid
DNPH – 2,4-dinitrophenyl hydrazine
DW - dry weight
EDTA – ethylenediaminetetraacetic acid disodium salt
FW - fresh weight
GSH – reduced glutathione
GSH-PX - glutathione peroxidase
GSSG – glutathione disulfide
GSSG-R – glutathione reductase
GST - glutathione S-transferase
H - hydroconditioning with water
$H_2O_2$ – hydrogen peroxide
HCl - hydrochloric acid
HMel 50 – hydroconditioning with MEL solutions at 50 μM
HMel 500 - hydroconditioning MEL solutions at 500 μM
HPLC-EC - High Pressure Liquid Chromatography with Electrochemical Detection
IAA – indole-3-acetic acid
MC – moisture content
MDA - malondialdehyde
MEL – melatonin
NBT - nitrotetrazolium blue chloride
NT - non-treated, control seeds
NADPH – β-nicotinamide adenine dinucleotide phosphate reduced form
NBT – nitrotetrazolium blue chloride
NO - nitric oxide
$OH^*$ - hydroxyl radical
$ONOO^-$ – peroxynitrite anion
PEG – polyethylene glycol
PMSF – phenylmethylsulfonyl fluoride
PVP – polyvinylpyrrolidone,
R – regeneration after stress
RNS - reactive nitrogen species
ROS - reactive oxygen species
S - chilling stress
SEM - standard error of the mean
SOD - superoxide dismutase
TBA – thiobarbituric acid
TBARS - thiobarbituric acid reactive substances
TCA – trichloroacetic acid and tween

## References

1. Yakhin, O.I.; Lubyanov, A.A.; Yakhin, I.A.; Brown, P.H. Biostimulants in Plant Science: A Global Perspective. *Front. Plant Sci.* **2017**, *7*, 2049.
2. Posmyk, M.M.; Szafrańska, K. Biostimulators: A new trend towards solving an old problem. *Front. Plant Sci.* **2016**, *7*, 748.
3. Soare, E.; Chiurciu, I.A.; Balan, A.V.; David L. World market research on maize. *Sciendo* **2018**, 216-222. DOI: 10.2478/alife-2018-0032
4. Ranum, P.; Pe˜na-Rosas, J.P.; Nieves Garcia-Casal, M. Global maize production, utilization, and consumption. *Ann. N.Y. Acad. Sci.* **2014**, *1312*, 105–112.
5. Guan, Y.; Hu, J.; Wang, X.; Shao, C. Seed priming with chitosan improves maize germination and seedling growth in relation to physiological changes under low temperature stress. *J Zhejiang Univ Sci B* **2009**, *10(6)*, 427-433.
6. Waqas, M.A.; Kaya, C.; Riaz, A.; Farooq, M.; Nawaz, I.; Wilkes, A.; Li, Y. Potential Mechanisms of Abiotic Stress Tolerance in Crop Plants Induced by Thiourea. *Front. Plant Sci.*, **2019**, *10*, 1336.

7. Devi, E.L.; Kumar, S.; Singh, B.; Sharma, S.K.; Beemrote, A. Adaptation Strategies and De-fence Mechanisms of Plants During Environmental Stress. *Medicinal Plants and Environmental Challenges* **2017**, 359-413.
8. Matsushima, K.I.; Sakagami, J.I. Effects of Seed Hydropriming on Germination and Seedling Vigor during Emergence of Rice under Different Soil Moisture Conditions. *Am. J. Plant Sci.* **2013**, *4,* 1584-1593.
9. Nakao, Y.; Asea, G.; Yoshino, M.; Kojima, N.; Hanada, H.; Miyamoto, K.; Yabuta, S.; Kamioka, R.; Sakagami, J.I. Development of Hydropriming Techniques for Sowing Seeds of Upland Rice in Uganda. *Am. J. Plant Sci.* **2018**, *9*, 2170-2182.
10. Ogbuehi, H.C.; Madukwe, D.K.; Ashilonu P. Assessment of hydro priming of seeds on performance of morphological indices of bambara groundnut (*Vigna subterrenea* linn.) landrace. *G.J B.A.H.S.* **2013**, *2(2)*, 17-22.
11. Aboutalebian, M.A.; Ekbatani, G.Z.; Sepehri, A. Effects of on-farm seed priming with zinc sulfate and urea solutions on emergence properties, yield and yield components of three rain fed wheat cultivars. *Ann. Biol. Res.* **2012**, *3(10)*, 4790-4796.
12. Jisha, K.C.; Vijayakumari, K.; Puthur, J.T. Seed priming for abiotic stress tolerance: an overview. *Acta Physiol. Plant* **2013**, *35*, 1381–1396.
13. Patel, R.V.; Pandya, K.Y; Jasrai, R.T.; Brahmbhatt, N. Effect of hydropriming and biopriming on seed germination of Brinjal and Tomato seed. Res. *J. Agriculture and Forestry Sci.*, **2017**, *5(6)*, 1-14.
14. Kołodziejczyk, I.; Posmyk, M.M. Melatonin – a new plant biostymulator? *J. Elementology* **2016**, *21(4)*, 1187-1198.
15. Janas, K.M.; Posmyk, M.M. Melatonin, an underestimated natural substance with great potential for agricultural application. *Acta Physiol. Plant.* **2013**, *35(12)*, 3285-3292.
16. Arnao, M.B.; Hernandez-Ruiz, J. Functions of melatonin in plants: a review. *J. Pineal Res.* **2015**, *59,* 133–150.
17. Tan, D.X.; Hardeland, R; Manchester, L.C.; Korkmaz, A; Ma, S; Rosales-Corral, S; Reiter, R.J. Functional roles of melatonin in plants, and perspectives in nutritional and agricultural science. *J. Exp. Bot.* **2011**, *63(2)*, 577–597.
18. Kocadağlı, T.; Yilmaz, C.; Gokmen, V. Determination of melatonin and its isomer in foods by liquid chromatography tandem mass spectrometry. *Food chemistry* **2014**, *153*, 151-156.
19. Pelagio-Flores, R.; Munoz-Parra, E.; Ortiz-Castro, R.; Lopez-Bucio, J. Melatonin regulates Arabidopsis root system architecture likely acting independently of auxin signaling. *J. Pineal Res.* **2012**, *53,* 279-288.
20. Stürtz, M.; Cerezo, A.B.; Cantos-Villar, E.; Garcia-Parrilla, M.C. Determination of the melatonin content of different varieties of tomatoes (*Lycopersicon esculentum*) and strawberries (*Fragariaananassa*). *Food Chem.* **2011**, *127(3)*, 1329-34.
21. Goodarzi, A.; Namdjoyan, S.; Soorki, A.A. Effects of exogenous melatonin and glutathione on zinc toxicity in safflower (*Carthamus tinctorius* L.) seedlings. *Ecotoxicology and Environmental Safety* **2020**, *201*, 110853.
22. Qiao, Y.; Ren, J.; Yin, L.; Liu, Y.; Deng, X.; Liu, P.; Wang, S. Exogenous melatonin alleviates PEG-induced short-term water deficiency in maize by increasing hydraulic conductance. *BMC Plant Biology* **2020**, *20*, 218.
23. Qiao, Y.; Yin, L.; Wang, B.; Ke, Q.; Deng, X.; Wang, S. Melatonin promotes plant growth by increasing nitrogen uptake and assimilation under nitrogen deficient condition in winter wheat. *Plant Physiol. And Biochem.* **2019**, *139*, 342-349.
24. Li, X.; Wei, J.P.; Scott, E.R.; Liu, J.W.; Guo, S.; Li, Y.; Zhang, L.; Han, W.Y. Exogenous melatonin alleviates cold stress by promoting antioxidant defense and redox homeostasis in *Camellia sinensis* L. *Molecules* **2018**, *23*, 165.
25. Arora, D.; Bhatla, S.C. Melatonin and nitric oxide regulate sunflower seedlings growth under salt stress accompanying differential expression of Cu/Zn SOD and Mn SOD. *Free Rad. Vio. Med.* **2017**, *106*, 315-328.
26. Byeon, Y.; Lee, H.Y.; Hwang, O.J.; Lee, H.J.; Lee, K.; Back, K. Coordinated regulation of melatonin synthesis and degradation genes in rice leaves in response to cadmium treatment. *J. Pineal Res.* **2015**; *58*, 470–478.
27. Shi, H.; Wang, X.; Tan, D.X.; Reiter, R.J.; Chan, Z. Comparative physiological and proteomic analyses reveal the actions of melatonin in the reduction of oxidative stress in Bermuda grass (*Cynodon dactylon* (L). Pers.) *J. Pineal Res.* **2015**, *59*, 120–131.
28. Rodrigues, C., Mayo, J.C., Sainz R.M. Regulation of antioxidant enzymes: A significant role for melatonin. *J. Pin. Res.* **2004,** *36(1)*,1-9.
29. Tan, D.X.; Manchester, L.C.; Terron, M.D.; Flores, L.J.; Reiter, R.J. One molecule, many derivatives, a never –ending interaction of melatonin with reactive oxygen and nitrogen species? *J. Pineal Res.* **2007**, *42*, 28–42.
30. Antoniou, C.; Chatzimichail, G.; Xenofontos, R; Pavlou, J.J.; Panagiotou, E.; Christou, A.; Fotopoulos, V. Melatonin systemically ameliorates drought stress-induced damage in Medicago sativa plants by modulating nitro-oxidative homeostasis and proline metabolism. *J. Pineal Res.* **2017**, *62*, 12401.
31. Meng, J.F.; Xu, T.F.; Wang, Z.Z.; Fang, Y.L.; Xi, Z.M.; Zhang, Z.W. The ameliorative effects of exogenous melatonin on grape cuttings under water-deficient stress: antioxidant metabolites, leaf anatomy, and chloroplast morphology. *J. Pineal Res.* **2014**, *57*, 200–212.
32. Zhao, D.; Wang, H.; Chen, S.; Yu, D.; Reiter, R.J. Phytomelatonin: An Emerging Regulator of Plant Biotic Stress Resistance. *Trends in Plant Sci.* **2020**, *2027*, 13.
33. Galano, A.; Tan, D.X.; Reiter, R.J. On the free radical scavenging activities of melatonin's metabolites, AFMK and AMK. *J. Pineal Res.* **2013**, *54*, 245–257.
34. Rastegar, S.; Khankahdani, H.H.; Rahimzadeh, M. Effects of melatonin treatment on the biochemical changes and antioxidant enzyme activity of mango fruit during storage. *Sci. Horticul.* **2020**, *259*, 108835.
35. Gao, Y.; Wang, Y.; Qian, J.; Si, W.; Tan, Q.; Xu, J.; Zhao, Y. Melatonin enhances the cadmium tolerance of mushrooms through antioxidant-related metabolites and enzymes. *Food Chem.* **2020**, *330*, 127263.

36. Lee, H.Y.; Byeon, Y.; Tan, D.X.; Reiter, R.j.; Back, K. Arabidopsis serotonin N-acetyltransferase knockout mutant plants exhibit decreased melatonin and salicylic acid levels resulting in susceptibility to an avirulent pathogen. J. Pineal Res. **2015**, *58*, 291–299.
37. Mayo, J.C.; Sainz, R.M.; Antolin, I.; Herrera, F.; Martin, V.; Rodrigues, C. Melatonin regulation of antioxidant enzyme gene expression. *Cell. Mol. Life Sci.* **2002**, *59*, 1706-1713.
38. Gao, H.; Lu, Z.; Yang, Y.; Wang, D.; Yang, T.; Cao, M.; Cao, W. Melatonin treatment reduces chilling injury in peach fruit throug its regulation of membrane fatty acid contents and phenotic metabolism. *Food Chem.* **2018**, *245*, 659-666.
39. Han, Q.H.; Huang, B.; Ding, C.B.; Zhang, Z.W.; Chen, Y.E.; Hu, C.; Zhou, L.J.; Huang, Y.; Liao, J.Q.; Yuan, S.; Yuan, M. Effects of melatonin on anti-oxidative systems and photosystem II in cold-stressed rice seedlings. *Front. Plant Sci.* **2017**, *8*, 785.
40. Liang, C.; Zheng, G.; Li, W.; Wang, Y.; Hu, B.; Wang, H.; Wu, H.; Qian, Y.; Zhu, X.G.; Tan, D.X.; Chen, S-Y.; Chu, C. Melatonin delays leaf senescence and enhances salt stress tolerance in rice. *J. Pineal Res.* **2015**, *59*, 91–101.
41. Reiter, R.J.; Rosales-Corral, S.; Tan, D.X.; Jou, M.J. Melatonin as a mitochondria-targeted antioxidant: one of evolution's best ideas. *Cell. Mol. Life Sci.* **2017**, *74*, 3863-3881.
42. Kołodziejczyk, I.; Dzitko, K.; Szewczyk, R.; Posmyk, M.M. Exogenous melatonin improves corn (*Zea mays* L.) embryo proteome in seeds subjected to chilling stress. *J. Plant Physiol.* **2016**, *193*, 47–56.
43. Taylor, A.G.; Allen, P.S.; Bennett, M.A. Seed enhancement. *Seed Sci. Res.* **1998**, *8*, 245-256.
44. Bailly, Ch.; El-Maarouf-Bouteau, H.; Corbineau, F. From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. *C. R. Biologies* **2008**, *331*, 806–814.
45. Posmyk, M.M.; Bałabusta, M.; Wieczorek, M.; Śliwińska, E.; Janas, K. Melatonin applied to cucumber (*Cucumis sativus* L.) seeds improves germination during chilling stress. *J. Pineal Res.* **2009**, *46*, 214-223.
46. Kołodziejczyk, I.; Bałabusta, M.; Szewczyk, R.; Posmyk M.M. The levels of melatonin and its metabolites in conditioned corn (*Zea mays* L.) and cucumber (*Cucumis sativus* L.) seeds during storage. *Acta Physiol. Plant.* **2015**, *37(6)*, 105.
47. Hattori, A., Migitaka, H., Iigo, M., Itoh, M., Yamamoto, K., Ohtani-Kaneko, R., Hara, M., Suzuki, T., Reiter, R.J. 1995. Identification of melatonin in plants and its effect on plasma melatonin levels and binding to melatonin receptors in vertebrates. *Biochem. Mol. Biol. Int.* **1995,** *35*, 627-634.
48. Park, S.; Lee, D.E.; Jang, H.; Byeon, Y.; Kim, Y.S.; Back, K. Melatonin rich transgenic rice plants exhibit resistance to herbicide-induced oxidative stress. *J. Pineal Res.* **2013**, *54*, 258–263.
49. Bajwa, V.S.; Shukla, M.R.; Sherif, S.M.; Murch, S.J.; Saxena, P.K. Role of melatonin in alle-viating cold stress in *Arabidopsis thaliana. J. Pineal Res.* **2014**, *56*, 238–245.
50. Zhang, N.; Sun, Q.; Zhang, H.; Cao, Y.; Weeda, S.; Ren, S.; Guo, Y.D. Roles of melatonin in abiotic stress resistance in plants. *J. Exp. Bot.* **2015**, *66(3)*, 647–656.
51. Hernandez-Ruiz, J.; Arnao, M.B. Melatonin stimulates the expansion of etiolated lupin cotyledons. *Plant Growth Regul.* **2008**, *55*, 29-34.
52. Wen, D.; Gong, B.; Sun, S.; Liu, S.; Wang, X.; Wei, M.; Yang, F.; Li, Y.; Shi, Q. Promoting roles of melatonin in adventitious root development of *Solanum licopersicum* L. by regulating auxin and nitric oxide signaling. *Front. Plant Sci.* **2016**, *7*, 718.
53. Chen, Q.; Qi, W.B.; Reiter, R.J.; Wei, W.; Wang, B.M. Exogenously applied melatonin stimulates root growth and raises endogenous IAA in roots of etiolated seedlings of *Brassica juncea*. *J. Plant Physiol.* **2009**, *166*, 324-328.
54. Li, T., Wu, Q., Zhu, H., Zhou, H., Jiang, Y., Gao, H., Yun, Z. Comparative transcriptomic and metabolic analysis reveals the effect of melatonin on delaying anthracnose incidence upon postharvest banana fruit peel. *BMC Plant Biology* **2019**, *19*, 289.
55. Zuo, B.; Zheng, X.; He, P.; Wang, L., Lei, Q., Feng, C., Zhou, J.; Li, Q.; Han, Z.; Kong, J. Overexpression of MzASMT improves melatonin production and enhances drought tolerance in transgenic *Arabidopsis thaliana* plants. *J. Pineal Res.* **2014**, *57*, 408-417.
56. Wang, L.Y.; Liu, W.X.; Wang, W.X.; Sun, Y. Exogenous melatonin improves growth and photosynthetic capacity of cucumber under salinity-induced stress. *Photosynthetica* **2016**, *54(1)*, 19-27.
57. Arnao, M.B.; Hernandez-Ruiz, J. The potential of phytomelatonin as a nutraceutical. *Molecules* **2018,** *23*, 238.
58. Liang, C.; Li, A.; Yu, H.; Li, W.; Liang, C.; Guo, S.; Zhang, R.; Chu, C. Melatonin Regulates Root Architecture by Modulating Auxin Response in Rice. *Front. Plant Sci.* **2017**, *8*, 134.
59. Cao, Q.; Li, G.; Cui, Z.; Yang, F.; Jiang, X.; Diallo, L.; Kong, F. Seed priming with melatonin improves the seed germination of waxy maize under chilling stress via promoting the antioxidant system and starch metabolism. *Sci. Rep.* **2019**, *9*, 15044.
60. Turk, H.; Erdal, S.; Genisel, M.; Atici, O.; Demir, Y.; Yanmis, D. The regulatory effect of melatonin on physiological, biochemical and molecular parameters in cold-stressed wheat seedlings. *Plant Growth Regul.* **2014**, *74*, 139-152.
61. Turk, H.; Erdal, S. Melatonin alleviates cold-induced oxidative damage in maize seedlings by up-regulating mineral elements and enhancing antioxidant activity. *J. Plant Nutr. Soil Sci.* **2015**, *178*, 433-439.
62. Zhang, H.; Liu, L.; Wang, Z.; Feng, G.; Gao, Q.; Li, X. Induction of low temperature tolerance in wheat by pre-soaking and parental treatment with melatonin. *Molecules* **2021**, *26*, 1192.
63. Yan, H.; Jia, S.; Mao, P. Melatonin priming alleviates aging-induced germination inhibition by regulating B-oxidation, protein translation, and antioxidant metabolism in oat (*Avena sativa* L.) seeds. *J. Mol. Sci.* **2020**, *21*, 1898.
64. Yin, Z.; Lu, J.; Meng, S.; Liu, Y.; Mostafa, I.; Qi, M.; Li, T. Exogenous melatonin improves salt tolerance in tomato by regulating photosynthetic electron flux and the ascorbate-glutathione cycle. *J. Plant Interactions* **2019**, *14(1)*, 453-463.
65. Bałabusta, M.; Szafrańska, K.; Posmyk, M.M. Exogenous melatonin improves antioxidant defence in cucumber seeds (Cucumis sativus L.) germinated under chilling stress. *Front. Plant Sci.* **2016**, *7*, 575.

66. Han, Q-H., Huang, B., Ding, C-B., Zhang, Z-W., Chen, Y-E., Hu, C., Zhou, L-J., Huang, Y., Liao, J-Q., Yuan, S., Yuan, M. Effects of Melatonin on Anti-oxidative Systems and Photosystem II in Cold-Stressed Rice Seedlings. *Front. Plant Sci*. **2017**, *8*, 785.
67. Zhang, N., Sun, Q., Zhang, H., Cao, Y., Weeda, S., Ren, S., Guo, Y.D. Roles of melatonin in abiotic stress resistance in plants. *J Exp Bot* **2015**, *66(3)*, 647–656.
68. Chevalier, C.; Nafati, M.; Mathieu-Rivet, E.; Bourdon, M.; Frangne, N.; Cheniclet, C.; Renaudin, J.P.; Gevaudant, F.; Hernould, M. Elucidating the functional role of endoreduplication in tomato fruit development. *Ann. Bot.* **2011**, *107*, 1159-1169.
69. Larkins, B.A.; Dilkes, B.P.; Dante, R.A.; Coelho, C.M.; Woo, Y.M., Liu, Y. Investigating the hows and whys of DNA endoreduplication. *J. Exp. Bot.* **2001**, *52*, 183–192.
70. De Veyler, L.; Larkin, J.C.; Schnittger, A. Molecular control and function of endoreplication in development and physiology. *Trends Plant Sci.* **2011**, *16(11)*, 624-34.
71. Kwiatkowska, M.; Popłońska, K.; Kaźmierczak, A.; Stępiński, D.; Rogala, K.; Polewczyk, K. Role of DNA endoreduplication, lipotubuloids, and gibberellic acid in epidermal cell growth guring fruit development of *Ornithogalum umbellatum. J. Exp. Bot.* **2007**, *58(8)*, 2023-2031.
72. Wang, Q.; An, B.; Shi, H.; Luo, H.; He, C. High Concentration of Melatonin Regulates Leaf Development by Suppressing Cell Proliferation and Endoreduplication in Arabidopsis. *Int. J. Mol. Sci.* **2017**, *18*, 991.
73. Tsumoto, Y.; Yoshizumi, T.; Kuroda, H.; Kawashima, M.; Ichikawa, T.; Nakazawa, M.; Yamamoto, N.; Matsui, M. Light-dependent polyploidy control by a CUE protein variant in Arabidopsis. *Plant Mol. Biol.* **2006**, *61*, 817-828.
74. Kudo, N.; Mii, M. Endoreduplication cycles during hypocotyl growth of cabbage (*Brassica oleracea* L.) under light and dark conditions. *Plant Biotechnol.* **2004**, *21*, 295-298.
75. Sugimoto-Shirasu, K.; Roberts, K. „Big it up": endoreduplication and cell-size control in plants. *Curr. Opin. Plant Biol.* **2003**, *6*, 544-553.
76. Gendreau, E.; Orbovic, V.; Höfte, H.; Traas, J. Gibberellin and ethylene control endoreduplication levels in the *Arabidopsis thaliana* hypocotyl. *Planta* **1999**, *209(4)*, 513-516.
77. Michael, B.E.; Kaufman, M.R. The osmotic potential of polyethylene glycol 6000. *Plant Physiol* **1973**; *51,* 914-916.
78. Hodges, M.D.; DeLong, J.M.; Forney, Ch.F.; Prange, R.K. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* **1999**, *207*, 604-611.
79. Lenz, A.G.; Costabel, U.; Shaltiel, S.; Cevine, R.L. Determination of carbonyl groups in oxidatively modified proteins by reduction with tritiated sodium borohydride. *Ann. Biochem.* **1989**, *177*, 419-425.
80. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dry binding. *Ann. Biochem.* **1976**, *72*, 248-254.
81. Levine, R.L.; Williams, J.A.; Stadtman, E.R.; Shacter, E. Carbonyl assays for determination of oxidatively modified proteins. *Methods in Enzymology* **1994**, *233*, 346-357.
82. Giannopolitis, C.N.; Ries, S.K. Superoxide dismutases. I. Occurence in higher plants. *Plant Physiol.* **1977**, *59*, 309-314.
83. Clairbone, A. Catalase activity. In: Greenwald RA, ed, Handbook of methods for oxygen radical research. Boca Ranton: *CRC Press* **1985**, 283-284.
84. Kato, M.; Shimizu, S. Chlorophyll metabolism in higher plants. VII. Chlorophyll degradation in senescing tobacco leaves; phenolic-dependent peroxidative degradation. *Can. J. Bot.* **1987**, *65*, 729-735.
85. Scebba, F.; Sebastiani, L.; Vitagliano, C. Activities of antioxidant enzymes during senescence of Prunus armeniaca leaves. *Biol. Plant.* **2001**, *44(1)*, 41-46.
86. Nagalakshmi, N.; Prasad, M.N.V. Responses of glutathione cycle enzymes and glutathione metabolism to copper stress in *Scenedesmus bijugatus*. *Plant Sci.* **2001**, *160*, 291-299.
87. Nakano, Y.; Asada, K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant & Cell Physiol.* **1981**, *22*, 867-880.
88. Esterbauer, H.; Grill, D. Seasonal variation of glutathione and glutathione reductase in needles of *Picea abies. Plant Physiol.* **1978**, *61*, 119-121.
89. Habig, W.H.; Pabst, M.J.; Jakoby, W.B. Glutathione S-transferases. The first enzymatic step in mercaptruric acid formation. *J. Biol. Chem.* **1974**, *249*, 7130-7139.
90. Byczkowska, A.; Kunikowska, A.; Kaźmierczak, A. Determination of ACC-induced cell-programmed death in roots of *Vicia faba* ssp. minor seedlings by acridine orange and ethidium bromide staining. *Protoplasma* **2013**, *250*, 121–128.
91. Kaźmierczak, A. Endoreplication in *Anemia phyllitidis* coincides with the development of gametophytes and male sex. *Physiol. Plant.* **2010**, *138*, 321–328.

Oświadczenia współautorów manuskryptu **Kołodziejczyk I., Kaźmierczak A., Posmyk M.M. (2021)** Melatonin application modifies antioxidant defence and induces endoreplication in maize seeds exposed to chilling stres. *International Journal of Molecular Science* (przyjęty do oceny) – wchodzącego w skład rozprawy doktorskiej:

| Imię i nazwisko | Szacunkowy udział [%] | Opis działań | Podpis |
|---|---|---|---|
| mgr Izabela Kołodziejczyk | 70 | Przygotowanie nasion: wybór techniki i opracowanie parametrów metody primingu; wykonanie hydrokondycjonowania (H, HMel50, HMel500). Testy kiełkowania i wzrostowe. Hodowla materiału roślinnego w ustalonych warunkach eksperymentu. Ocena uszkodzeń oksydacyjnych. Analizy aktywności enzymów antyoksydacyjnych oraz detoksykujących. Przygotowanie i utrwalenie materiału do analiz zawartości DNA w jądrach komórkowych. Pomiary densytometryczne. Analiza i interpretacja wyników. Przygotowanie manuskryptu. | Kołodziejczyk Izabela |
| dr hab. Andrzej Kaźmierczak, prof. UŁ | 15 | Przygotowanie eksperymentów i ich metodyki, związanych z barwieniem DAPI oraz usytuowaniem komórek w określonych fazach cyklu komórkowego. Zebranie i opracowanie wyników pomiarów densytometrycznych. | |
| prof. dr hab. Małgorzata M. Posmyk | 15 | Współtwórca koncepcji pracy. Pomoc metodyczna na różnych etapach analiz. Obliczenia statystyczne. Pomoc w interpretacji wyników i redakcji manuskryptu. | |
| Manuskrypt przyjęty do oceny dn. 25.05.2021 r. IF 4.556[2019], 4.653[V] (140[A] pkt. MEiN) | | | |

User Menu

Home (/user/myprofile)
Manage Accounts (/user/manage_accounts)
Change Password (/user/chgpwd)
Edit Profile (/user/edit)
Logout (/user/logout)

Submissions Menu

Submit Manuscript (/user/manuscripts/upload)
Display Submitted Manuscripts (/user/manuscripts/status)
English Editing (/user/pre_english_article/status)
Discount Vouchers (/user/discount_voucher)
Invoices (/user/invoices)
LaTex Word Count (/user/get/latex_word_count)

Reviewers Menu

Volunteer Preferences (/volunteer_reviewer_info/view)

# Manuscript Information Overview

| | |
|---|---|
| Manuscript ID | **ijms-1252096** |
| Status | Under review |
| Article type | Article |
| Title | Melatonin application modifies antioxidant defense and induces endoreplication in maize seeds exposed to chilling stress |
| Journal | *International Journal of Molecular Sciences* (https://www.mdpi.com/journal/ijms) |
| Section | Molecular Plant Sciences (https://www.mdpi.com/journal/ijms/sections/molecular_plant_sciences) |
| Special Issue | The Role of Melatonin in Plants (https://www.mdpi.com/journal/ijms/special_issues/Melatonin_Plants) |

**Abstract** The work concerns biostimulating properties of melatonin (MEL) applied to maize seeds by hydroconditioning. For the tests at the physiological, biochemical and cytological level, control, non-treated seeds (NT), hydroconditioned with pure water (H) and with aqueous MEL solutions at 50 and 500 µM concentrations (HMel 50 and HMel 500, respectively) were used. It was demonstrated that the application of exogenous MEL to seeds improved their germination under suboptimal temperatures and stimulated the growth of embryonic axes under chilling stress conditions (5 oC) and after stress removal, during regeneration. The antioxidant activity of MEL was confirmed by low level of protein oxidative damage and smaller quantity of lipid oxidation products in embryonic axes of HMel 50 and HMel 500 seeds exposed to chilling temperature 5 oC for 14 days. The activity of basic antioxidant and detoxifying enzymes was also analyzed. The stimulatory effects of MEL on SOD, peroxidases: APX and GSH-PX and on GST, a detoxifying enzyme were demonstrated. It was also showed, for the first time, that MEL induced defense strategies against stress at the cytological level, inducing endoreplication in embryonic axes cells partially even in the seeds germinating under optimal conditions (preventive action), but very intensively in those germinating under chilling stress conditions (intervention action), and after stress removal, to improve regeneration.

**Keywords** antioxidant enzymes; biostimulation; chilling stress; corn seeds; endoreplication; melatonin; seed priming; Zea mays

| | |
|---|---|
| Manuscript File | manuscript.docx (/user/manuscripts/displayFile/475db491e53eab3cc7e7c911eb4a033e) |
| PDF File | manuscript.pdf (/user/manuscripts/displayFile/475db491e53eab3cc7e7c911eb4a033e/latest_pdf) |

## Author Information


| | |
|---|---|
| Submitting Author | Izabela Kołodziejczyk |
| Corresponding Author | Izabela Kołodziejczyk |
| Author #1 | Izabela Kołodziejczyk |
| E-Mail | izabela.kolodziejczyk@biol.uni.lodz.pl |
| Author #2 | Andrzej Kaźmierczak |
| E-Mail | andrzej.kazmierczak@biol.uni.lodz.pl |

Author #3 Małgorzata M. Posmyk

E-Mail malgorzata.posmyk@biol.uni.lodz.pl

## Manuscript Information

| | |
|---|---|
| Received Date | 24 May 2021 |
| Page Count | 26 |

## APC information

| | |
|---|---|
| Journal APC: | 2,000.00 CHF |
| IOAP Participant: | University of Lodz |
| IOAP Payment: | Non-central: Invoiced to author |
| Author Eligible Central: | No |
| IOAP Discount: | 10% |
| Total Payment Amount: | 1,800.00 CHF |

## Related Papers Published in MDPI Journals

If you have any questions or concerns, please do not hesitate to contact ijms@mdpi.com (mailto: ijms@mdpi.com).

*Załącznik 6*

# Web of Science

Clarivate Analytics



## Citation report for 4 results from All Databases between 1864 and 2021 Go

You searched for: AUTHOR: (Kolodziejczyk I) ...More

This report reflects citations to source items indexed within All Databases.

Export Data: Save to Excel File

**Total Publications**
4 Analyze

1997 2016

***h*-index**
4

**Average citations per item**
18,25

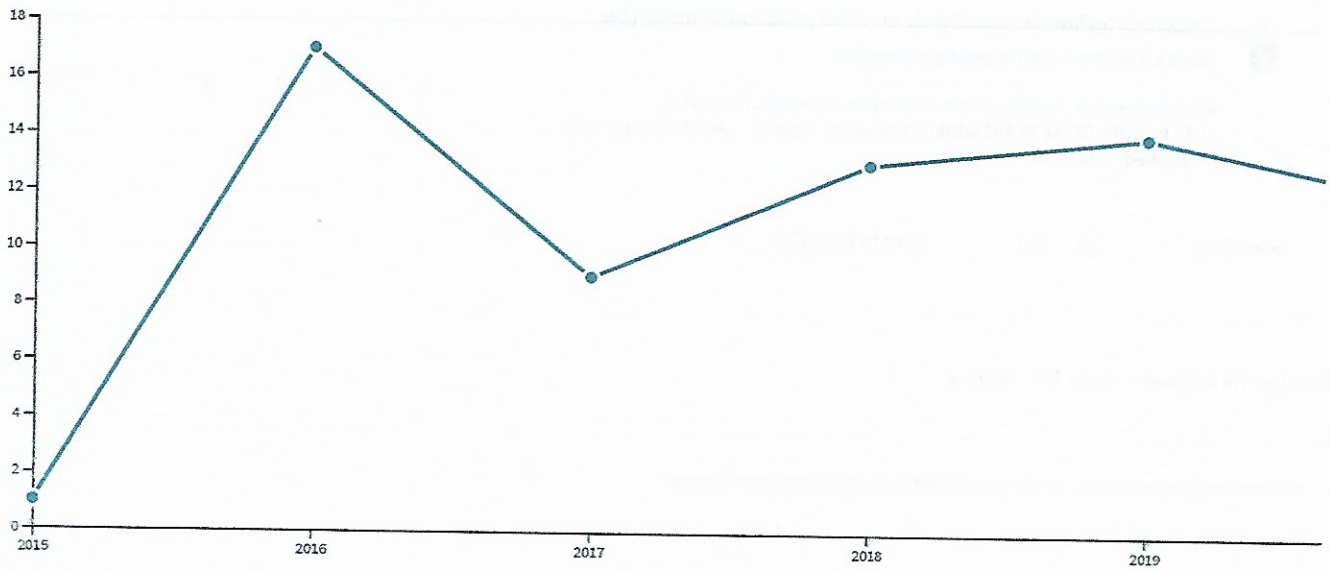
**Sum of Times Cited**
73

**Without self citations**
71

**Citing articles**
55 Analyze

**Without self citations**
53 Analyze

Sum of Times Cited per Year



Sort by: Times Cited Date More

1 of 1

How are these totals calculated?

| | | 2017 | 2018 | 2019 | 2020 | 2021 | Total | Average Citations per Year |
|---|---|---|---|---|---|---|---|---|
| Use the checkboxes to remove individual items from this Citation Report | | 9 | 13 | 14 | 12 | 7 | 73 | 10.43 |
| or restrict to items published between 1864 and 2021 Go | | | | | | | | |
| 1. | The levels of melatonin and its metabolites in conditioned corn (Zea mays L.) and cucumber (Cucumis sativus L.) seeds during storage<br>By: Kolodziejczyk, Izabela; Balabusta, Marta; Szewczyk, Rafal; et al.<br>**ACTA PHYSIOLOGIAE PLANTARUM** Volume: 37 Issue: 6 Article Number: 105 Published: JUN 2015 | 1 | 3 | 2 | 3 | 0 | 17 | 2.43 |
| 2. | MELATONIN - A NEW PLANT BIOSTIMULATOR?<br>By: Kolodziejczyk, Izabela; Posmyk, Malgorzata M.<br>**JOURNAL OF ELEMENTOLOGY** Volume: 21 Issue: 4 Pages: 1187-1198 Published: 2016 | 1 | 2 | 4 | 2 | 1 | 14 | 2.33 |
| 3. | Exogenous melatonin improves corn (Zea mays L.) embryo proteome in seeds subjected to chilling stress<br>By: Kolodziejczyk, Izabela; Dzitko, Katarzyna; Szewczyk, Rafal; et al.<br>**JOURNAL OF PLANT PHYSIOLOGY** Volume: 193 Pages: 47-56 Published: APR 1 2016 | 5 | 4 | 6 | 6 | 4 | 30 | 5.00 |
| 4. | Exogenous melatonin expediently modifies proteome of maize (Zea mays L.) embryo during seed germination<br>By: Kolodziejczyk, Izabela; Dzitko, Katarzyna; Szewczyk, Rafal; et al.<br>**ACTA PHYSIOLOGIAE PLANTARUM** Volume: 38 Issue: 6 Article Number: 146 Published: JUN 2016 | 2 | 4 | 2 | 1 | 2 | 12 | 2.00 |

Select Page Save to Excel File

Sort by: Times Cited Date More

1 of 1

*4 records matched your query of the 134,990,994 in the data limits you selected.*