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Eliminacja herbicydów chloroacetanilidowych przez wybrane grzyby strzępkowe z rodzaju *Trichoderma*

Elimination of chloroacetanilide herbicides by selected filamentous fungi of the genus *Trichoderma*

Praca doktorska

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1. **Nykiel-Szymańska J.**, Bernat P., Słaba M. (w druku). Biotransformation and detoxification of chloroacetanilide herbicides by *Trichoderma* spp. with plant growth-promoting activities. Pesticide Biochemistry and Physiology; 10.1016/j.pestbp.2019.11.018

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2. **Nykiel-Szymańska J.**, Bernat P., Słaba M. **2018**. Potential of *Trichoderma koningii* to eliminate alachlor in the presence of copper ions. Ecotoxicology and Environmental Safety 162, 1-9; 10.1016/j.ecoenv.2018.06.060

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3. **Nykiel-Szymańska J.**, Różalska S., Bernat P., Słaba M. **2019**. Assessment of oxidative stress and phospholipids alterations in chloroacetanilides-degrading *Trichoderma* spp.. Ecotoxicology and Environmenral Safety 184; 10.1016/j.ecoenv.2019.109629

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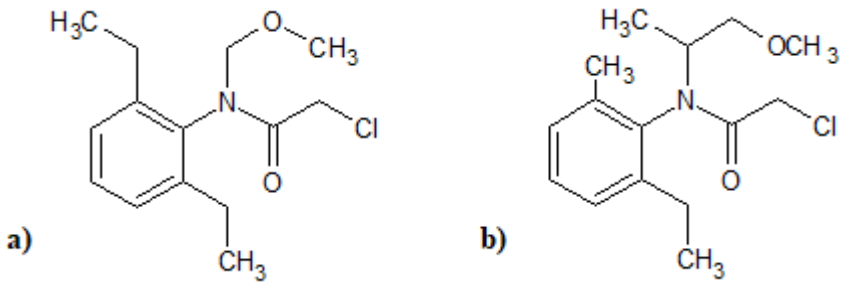
I Wprowadzenie

Powszechne stosowanie chemicznych środków ochrony roślin przyczyniło się do rozprzestrzenienia zanieczyszczeń organicznych w środowisku rolniczym. Obecnie na świecie rocznie zużywa się 2,5 mln ton pestycydów, z czego największą część stanowią herbicydy (ok. 40%) (Fenner i in., 2013). Tylko minimalna część stosowanego pestycydu trafia do organizmu docelowego (0,1%), podczas gdy reszta ulega rozproszeniu w środowisku, zanieczyszczając glebę, wody powierzchniowe i gruntowe, stanowiąc tym samym zagrożenie dla organizmów żywych (Olchanheski i in., 2014).

Alachlor [2-chloro-N-2,6-diethylphenyl-N-(methoxymethyl)acetamid] i metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamid] należą do grupy herbicydów chloroacetanilidowych, powszechnie stosowanych w rolnictwie ze względu na dużą skuteczność w eliminacji jednorocznych traw, turzyc oraz chwastów liściastych w uprawach soi, kukurydzy, bawełny, słoneczników, rzepaku czy orzeszków ziemnych (**Rys. 1**) (Huang i in., 2017). Chloroacetanilidy są selektywnymi herbicydami o działaniu systemowym, wchłanianymi przez kiełkujące pędy i korzenie roślin docelowych. Mechanizm działania opiera się na zahamowaniu biosyntezy białek, a także kwasów tłuszczowych o długich łańcuchach węglowych, co w konsekwencji prowadzi do zatrzymania wzrostu roślin (Souissi i in., 2013).



Rys. 1 Wzory strukturalne alachloru (**a**) oraz metolachloru (**b**).

Ze względu na stosunkowo dobrą rozpuszczalność w wodzie (alachlor 242 mg/L w 20°C; metolachlor 530 mg/L w 20°C) oraz niski stopień mineralizacji, substancje te przedostają się do wód powierzchniowych i gruntowych, gdzie wiążą się zarówno ze składnikami gleby jak i składnikami osadów dennych (Huang i in., 2017). Dane literaturowe wskazują, iż rozkład chloroacetanilidów w warunkach środowiskowych pod wpływem

czynników atmosferycznych przebiega z niską wydajnością, co w rezultacie prowadzi do akumulacji tych związków oraz ich pochodnych w glebie, rzekach, morzach, a także wodzie pitnej (Zhang i in., 2011). Komisja Europejska ustaliła dopuszczalny poziom zawartości alachloru w wodzie pitnej - 0,1 µg/L, natomiast suma zawartości herbicydu oraz jego metabolitów nie może przekraczać 0,5 µg/L wody. Natomiast według Amerykańskiej Agencji Ochrony Środowiska (EPA) dozwolony maksymalny poziom alachloru w wodzie pitnej wynosi 2,0 µg/L (Qiang i in., 2010). W wyniku nadmiernego oraz niekontrolowanego stosowania herbicydów, substancje te stanowią realne zagrożenie dla środowiska naturalnego oraz organizmów niestanowiących celu zwalczania dla środków chwastobójczych. Badane herbicydy są wysoce toksyczne dla organizmów wodnych, roślin oraz zielonych alg (Maronić i in., 2018). Na podstawie badań prowadzonych na zwierzętach EPA sklasyfikowała alachlor w grupie B2 czynników kancerogennych jako prawdopodobnie rakotwórczy dla ludzi. Natomiast metolachlor jest pestycydem należącym do kategorii C, która charakteryzuje się znikomym prawdopodobieństwem rakotwórczego działania (US EPA, 2017). Chloroacetanilidy mogą ulegać akumulacji w tkankach, co stanowi szczególne zagrożenie dla człowieka, będącego ostatnim ogniwem łańcucha pokarmowego. Zarówno alachlor jak i metolachlor wykazują właściwości ksenoestrogenne, tzn. zakłócają prawidłowe funkcjonowanie układu hormonalnego ludzi i zwierząt. Alachlor wiąże się kompetencyjnie z receptorami żeńskich hormonów płciowych. Ponadto alachlor i metolachlor poprzez aktywację receptora hormonów steroidowych (pregnan X) zakłócają syntezę enzymów odpowiedzialnych za ich metabolizm (Mnif i in., 2011). Szkodliwe działanie herbicydów może być również związane z generowaniem reaktywnych form tlenu (RFT), co prowadzi do utraty równowagi pomiędzy przeciwutleniaczami a utleniaczami, a w konsekwencji może powodować oksydacyjne uszkodzenia białek, lipidów czy kwasów nukleinowych (Jiang i in., 2016; Sies i in., 2017). Na tej podstawie Unia Europejska uchwaliła Dyrektywę 2008/105/EC na mocy której od 2008 alachlor został wycofany z użytku na terenie UE oraz został wpisany na listę substancji szczególnie niebezpiecznych dla środowiska wodnego, które powinny zostać całkowicie z niego wyeliminowane. Natomiast metolachlor jest dopuszczony do użytku na terenie krajów Unii Europejskiej. Poza Europą alachlor wciąż jest powszechnie stosowany w praktyce rolniczej, zwłaszcza w USA, Indiach, Republice Południowej Afryki, Chinach i Japonii, a także w Bangladeszu, Indonezji, Malezji, Nepalu, Pakistanie, Filipinach,

Singapurze, Sri Lance, Tajlandii i Wietnamie (Stamper i Tuovinen, 1998; FAO of the United Nations Regional Office for Asia and the Pacific, 2013).

Zastosowanie konwencjonalnych fizykochemicznych metod usuwania chloroacetanilidów z różnych środowisk obarczone jest m.in. wysokimi kosztami, niską wydajnością procesu, wytwarzaniem toksycznych metabolitów lub minimalnym zmniejszeniem toksyczności herbicydów (Souissi i in.,2013; Qiang i in., 2010). Mikrobiologiczna degradacja uważana jest za najważniejszą drogę transformacji alachloru i metolachloru w środowisku naturalnym, a kluczową rolę w tym procesie przypisuje się mikroorganizmom wyizolowanym ze skażonych środowisk, które charakteryzują się zdolnością adaptacji do warunków środowiska, a także możliwością wykorzystywania szeregu związków organicznych jako substratów energetycznych i budulcowych (Huang i in., 2017). Źródła literaturowe podają szereg mikroorganizmów zdolnych do transformacji chloracetanilidów zarówno w warunkach tlenowych jak i beztlenowych. Jednakże większość badań dotyczy wykorzystania szczepów bakteryjnych z rodzaju *Agrobacterium* sp., *Ancylobacter* sp., *Burkholderia* sp. (Ewida, 2014), *Bacillus* sp. (Wang i in., 2008; Xu i in., 2008), *Microbacterium* sp., *Pseudomonas* sp. (Xu i in., 2008) oraz *Streptomyces* sp. (Durães Sette i in., 2004). Mniej natomiast wiadomo na temat możliwości metabolizowania alachloru i metolachloru przez grzyby strzępkowe. Badania prowadzono głównie na modelu grzybów zgnilizny drewna np. *Chaetomium globosum* (Tiedje i Hagedorn, 1975) *Phanerochaete chrysosporium* (Ferrey i in., 1994; Chirnside i in., 2009) i *Coriolus versicolor* (Hai i in., 2012), a także z wykorzystaniem mikroskopowych grzybów strzępkowych np. *Cunninghamella elegans* (Pothuluri i in., 1993, 1997), *Paecilomyces marquandii* (Słaba i in., 2013) oraz drożdży *Candida xestobii* (Munoz i in., 2011).

Grzyby z rodzaju *Trichoderma* sp. są kosmopolitycznymi mikroorganizmami, izolowanymi najczęściej z gleby oraz korzeni roślin. Powszechność ich występowania wynika ze zdolności do szybkiego wzrostu i przeżycia w niekorzystnych warunkach, a także możliwości wykorzystania różnych substratów jako źródła węgla i azotu (Tripathi i in., 2013). Grzyby te znane są przede wszystkim jako drobnoustroje, które wspomagają wzrost roślin i chronią je przed patogenami (Raspanti i in., 2009). Przez wiele lat uważano, iż promowanie wzrostu przez *Trichoderma* sp. jest bezpośrednim wynikiem wytwarzania metabolitów wtórnych (kwas harzianowy, tricholin, kwas heptelidowy, wirydyna, peptaibole, gliowiryna,

massoilakton, gliotoksyna, alamentycyny, 6-pentyl-a-pyrol, glisopreniny), które wykazują antagonistyczne działanie w stosunku do fitopatogenów. Kolejne badania wykazały, że stymulacja wzrostu roślin jest wynikiem oddziaływania synergistycznych mechanizmów, m.in. syntezy fitohormonów wzrostu (np. kwasu indolilo-3-octoweg, kwas giberelinowego), zwiększenia biodostępności makro- i mikroskładników w glebie (np. fosforu, żelaza, miedzi, cynku i manganu), rozwoju strefy korzeniowej, a także stymulacją tempa metabolizmu węglowodanów oraz indukcją odporności roślin na stres abiotyczny i biotyczny (Stewart i Hill, 2014). Grzyby z rodzaju *Trichoderma w*ykazują także oporność w stosunku do wielu toksycznych związków, np. środków ochrony roślin, metali ciężkich i związków metaloorganicznych. Wysoka aktywność metaboliczna sprawia, iż wyselekcjonowane szczepy *Trichoderma* są zdolne do degradacji wielu związków chemicznych, np. cyjanków, krezoli, fenoli, aromatycznych amin, zieleni malachitowej, WWA, a także środków ochrony roślin, np. kwasu 2,4-dichlorofenoksyoctowego (2,4-D), 2,4-dichlorofenolu (2,4-DCP), pentachlorofenolu (PCP), dichlorodifenylotrichloroetanu (DDT), dichlorfosu, chloropiryfosu (Blaya i in., 2013; Tripathi i in., 2013). Te właściwości sprawiają, że *Trichoderma* są szczególnie interesującym obiektem badań, zwłaszcza dla potencjalnego zastosowania w bioremediacji skażonych gleb i wód.

Niniejsza praca doktorska dotyczy oceny zdolności wybranych szczepów mikroskopowych grzybów strzępkowych z rodzaju *Trichoderma*: *T. atroviride* IM QF10, *T. hamatum* IM I-1, *T. harzianum* IM 0961, *T. koningii* IM 0956, *T. citrinoviride* IM 6325, *T. harzianum* KKP 534, *T. viride* KKP 792 oraz *T. virens* DSM 1963 do eliminacji alachloru i metolachloru. Jako model badawczy wybrano szczepy wyizolowane z różnych lokalizacji geograficznych (Polski, Rosji, Węgier, USA) i zdeponowane w różnych kolekcjach drobnoustrojów. Szczepy *T. atroviride* IM QF10, *T. hamatum* IM I-1, *T. citrinoviride* IM 6325, *T. harzianum* IM 0961 oraz *T. koningii* IM 0956 należą do kolekcji Katedry Mikrobiologii Przemysłowej i Biotechnologii Uniwersytetu Łódzkiego. *T. harzianum* KKP 534 i *T. viride* KKP 792 pochodzą z Kolekcji Kultur Drobnoustrojów Przemysłowych (IBPRS) w Warszawie, natomiast *T. virens* DSM 1963 z Niemieckiej Kolekcji Mikroorganizmów i Hodowli Komórkowych (DSMZ). Wcześniejsze badania wykazały zdolność *T. citrinoviride* IM 6325 do efektywnej eliminacji pentachlorofenolu (Szewczyk i in., 2003), a także *T. virens* DSM 1963 do biodegradacji tworzyw sztucznych (Klausmeier, 1972). Natomiast *T. harzianum* IM 0961

stymulował wzrost siewek pszenicy w obecności kwasu 2,4-dichlorofenoksyoctowego (Bernat i in., 2018a). Ponadto przesłanką do podjęcia badań były również prace dotyczące zdolności szczepów *T. virens* oraz *T. reesei* do efektywnej eliminacji anilin i chloroanilin (m.in. 3-chloro-4-metyloanilina (3,4-CMA), 2,6-dietyloanilina (2,6-DEA), 2-etylo-6-metyloanilina (2,6-EMA), 3,4-dichloroanilina (3,4-DCA)), które są produktami transformacji wielu herbicydów anilidowych (Cocaign i in., 2013).

II Cele pracy

Za główne cele badań przeprowadzanych podczas realizacji pracy doktorskiej przyjęto:

1. Analiza zdolności wybranych szczepów z rodzaju *Trichoderma* do biotransformacji alachloru i metolachloru.
2. Ocena wpływu wybranych szczepów *Trichoderma* na wzrost siewek rzepaku poddanych ekspozycji na badane herbicydy chloroacetanilidowe.
3. Badanie oddziaływania herbicydów na szczepy *Trichoderma* zdolne do biotransformacji chloroacetanilidów oraz analiza zmian adaptacyjnych indukowanych obecnością tych związków.

III Realizacja poszczególnych celów pracy

III.1 Analiza zdolności wybranych szczepów z rodzaju *Trichoderma* do biotransformacji alachloru i metolachloru.

W pierwszym etapie badań oceniono zdolność ośmiu szczepów grzybów strzępkowych z rodzaju *Trichoderma: T. atroviride* IM QF10, *T. hamatum* IM I-1, *T. harzianum* IM 0961, *T. koningii* IM 0956, *T. citrinoviride* IM 6325, *T. harzianum* KKP 534, *T. viride* KKP 792 oraz *T. virens* DSM 1963 do biotransformacji alachloru i metolachloru. Wykazano iż, wszystkie badane mikroorganizmy charakteryzują się wysokim potencjałem degradacyjnym zarówno alachloru (50 mg/L) jak i metolachloru (50 mg/L), ale różnią się pod względem tempa eliminacji tych związków. Najszybszy ubytek alachloru odnotowano dla pięciu szczepów *Trichoderma* spp.: IM QF10, IM 6325, IM I-1, IM 0961 oraz IM 0956, które podczas początkowych 72 h eliminowały około 80-90% początkowego stężenia herbicydu. Natomiast po 168 h inkubacji odnotowano minimalną zawartość alachloru (ok.1%). Szczepy DSM 1963 oraz KKP 792 charakteryzują się najniższą wydajnością eliminacji alachloru, po 7 dniach hodowli stwierdzono odpowiednio 80 i 89% ubytek zawartości ksenobiotyku. W przypadku hodowli *Trichoderma* sp. z dodatkiem metolachloru zaobserwowano wolniejsze tempo degradacji herbicydu niż w przypadku alachloru. Najefektywniejsza eliminacja wystąpiła pomiędzy 72 a 120 h inkubacji. W hodowlach IM 0956, IM 0961, IM QF10, IM I-1, IM 6325, KKP 534 oraz DSM 1963 odnotowano 62-79% eliminację metolachloru, natomiast szczep KKP 792 powodował ubytek jedynie 40% substratu. Prawdopodobną przyczynę różnic w tempie biotransformacji alachloru i metolachloru można upatrywać w strukturze tych związków. Według danych literaturowych chloroacetanilidy posiadające łańcuch alkoksymetylowy zlokalizowany przy azocie amidowym (m.in. alachlor, acetochlor, butachlor) są szybciej degradowane niż herbicydy z łańcuchem alkoksyetylowym przy azocie amidowym (m.in. metolachlor, pretilachlor) (Hou i in., 2014; Zhang i in., 2011). Analizy widm masowych uzyskanych poprzez zastosowanie wysokosprawnej chromatografii cieczowej sprzężonej z tandemową spektrometrią mas umożliwiły identyfikację 8 metabolitów alachloru oraz 4 metabolitów metolachloru, a dwa z nich zostały po raz pierwszy zidentyfikowane w hodowli mikroskopowych grzybów strzępkowych. Biotransformacja alachloru przez badane szczepy *Trichoderma* rozpoczyna się od reakcji dechlorynacji, a następnie podstawniki N-alkilowe ulegają reakcjom hydroksylacji, co w konsekwencji prowadzi do powstania mono-, di- lub

trihydroksylowanych produktów ubocznych. Natomiast metabolity metolachloru powstają głównie na drodze O-demetylacji podstawnika N-alkilowego oraz na drodze dechlorynacji. Biotransformacja jest najważniejszą drogą usuwania ksenobiotyków ze środowiska, jednakże może prowadzić do powstawania oraz akumulacji toksycznych i kancerogennych metabolitów pośrednich. Z tego powodu, między innymi, zabroniono w Europie aplikacji alachloru. Ocena toksyczności produktów mikrobiologicznej transformacji alachoru i metolachloru została wykonana z użyciem słonowodnego skorupiaka *Artemia franciscana* (test Artoxkit M). Wyniki testu Artoxkit M wskazują na niemal całkowitą detoksykację alachloru w hodowlach *T. harzianum* IM 0961, *T. koningii* IM 0956 oraz *T. hamatum* IM I-1. Z kolei w hodowlach KKP 534, KKP 792 i DSM 1963 zahamowanie ruchliwości larw *A. fransciscana* wynosiło 20-50% w porównaniu do 100% immobilizacji skorupiaków po 24 h inkubacji z alachlorem w stężeniu 50 mg/L. Badanie toksykologiczności ekstraktów pohodowlanych IM I-1, IM 0956, IM 0961, KKP 534 i KKP 792 inkubowanych z dodatkiem metolachloru wykazało 1,5-2,5-krotną redukcję toksyczności herbicydu w porównaniu z kontrolą abiotyczną. Uzyskane wyniki wyraźnie wskazują na spadek toksyczności zarówno alachloru jak i metolachloru przez badane szczepy *Trichoderma*. W ekstraktach pochodzących z hodowli IM QF10 i IM 6325 nietraktowanych herbicydami zaobserwowano wysoką śmiertelność *A. franciscana*, co może mieć związek z wytwarzaniem metabolitów wtórnych o aktywności przeciwdrobnoustrojowej oraz ich szkodliwym wpływie na wodne bezkręgowce m.in. *A. franciscana* i *Daphnia magna* (Favilla i in., 2006; Thiruchchelvan i in., 2013). Ponadto w płynach pohodowlanych wyżej wymienionych szczepów grzybowych zidentyfikowano metabolity wtórne o działaniu przeciwdrobnoustrojowym, takie jak kwas harzianowy, kwas harziafilowy, T22azaphilon, T39butenolid i trichoharzin. Obecność tych związków, choć utrudnia interpretacje testów toksyczności, może chronić rośliny uprawne przed fitopatogenami.

Wyniki badań mające na celu ocenę zdolności ośmiu wybranych szczepów *Trichoderma* do eliminacji popularnych chloroacetanilidów - alachloru i metolachloru, jakościową analizę powstałych produktów mikrobiologicznej transformacji, a także ocenę ich toksyczności zostały opisane w pracy oryginalnej "Biotransformation and detoxification of chloroacetanilide herbicides by *Trichoderma* spp. with plant growth-promoting activities"

(Pesticide Biochemistry and Physiology; 10.1016/j.pestbp.2019.11.018; IF= 3.44, 100 pkt MNiSW).

Realizację pierwszego celu pracy doktorskiej kontynuowano poddając ocenie udział lakazy oraz cytochromu P450, w eliminacji alachloru przez szczep *T. koningii* IM 0956. Szczep ten został wybrany, ponieważ oprócz dużej aktywności w eliminacji alachloru był zdolny do utleniania aminobenzotriazolu (ABTS), co świadczy o wytwarzaniu lakazy, a także charakteryzował się wysoką tolerancją na miedź. Badania dotyczące niespecyficznych enzymów oksydacyjnych - lakazy oraz monooksygenazy cytochromu P450 wskazują na ich istotną rolę w metabolizmie ksenobiotyków, m.in.: alachloru (Chirnside i in., 2011; Pothuluri i in., 1993), bisfenolu A (Yang i in., 2013), 2,4-D (Nykiel-Szymańska i in., 2017), dibutylocyny (Siewiera i in., 2017) lub zieleni malachitowej (Jasińska i in., 2015).

Lakaza jest enzymem oksydoredukcyjnym charakterystycznym dla grzybów białej zgnilizny, niemniej jednak potwierdzono zdolność do wytwarzania tego enzymu u niektórych grzybów z rodzaju *Trichoderma*: *T. atroviride* (Chakroun i in., 2010), *T. aureoviridae* (Khambhaty i in., 2015), *T. harzianum* (Sadhasivam i in., 2008) oraz *T. koningii* (Wang i in., 2012). Spośród ośmiu badanych szczepów *Trichoderma* tylko szczep *T. koningii* IM 0956 charakteryzował się aktywnością lakazy w hodowlach zarówno bez jak i z dodatkiem alachloru. Dodatek jonów miedzi do pożywki wzrostowej badanego szczepu znacząco stymulował aktywność lakazy w porównaniu do układu kontrolnego (0,02-0,42 U/L dla kontroli, 0,05-13,07 U/L dla prób badanych), co potwierdza dane literaturowe, iż dodatek Cu (II), jest niezbędny do wzmożonej aktywności lakazy (Chakroun i in., 2010; Khambhaty i in., 2015). Najwyższą aktywność enzymu uzyskano w obecności 7,5 mM miedzi. Ponadto wykazano, że wysoka aktywność lakazy występuje przy jednoczesnej suplementacji hodowli *T. konigii* jonami miedzi oraz alachlorem.

Następnie poddano ocenie udział enzymów oksydoredukcyjnych w procesie biotransformacji alachloru przez *T. koningii* poprzez analizę kinetyki rozkładu herbicydu w obecności inhibitorów lakazy (azydku sodu) oraz cytochromu P450 (aminobenzotriazolu i proadifenu). Dodatek azydku sodu do hodowli IM 0956 spowodował niewielkie, aczkolwiek istotne statystycznie zahamowanie tempa degradacji chloroacetanilidu przez cały okres trwania hodowli, co potwierdza udział lakazy w biotransformacji herbicydu przez IM 0956.

Natomiast obecność inhibitorów cytochromu P450 znacząco spowolniła tempo rozkładu alachloru podczas pierwszych 24 h hodowli, kiedy proces eliminacji ksenobiotyku zachodzi najintensywniej. Z kolei w późniejszych etapach hodowli wpływ inhibitorów był mniejszy, ale wciąż istotny statystycznie. Uzyskane wyniki pozwalają stwierdzić, iż biotransformacja alachloru przez *T. koningii* jest złożonym procesem obejmującym udział cytochromu P450 oraz lakazy. Wybrany szczep charakteryzował się również tolerancją na obecność jonów miedzi (1,0-7,5 mM). Związki miedzi są nie tylko induktorem aktywności lakazy, ale również są często stosowane w ochronie roślin jako fungicydy (Filimonova i in., 2018). W związku z tym przeanalizowano również kinetykę rozkładu alachloru przez *T. koningii* w obecności tego metalu ciężkiego. W obecności 1 mM miedzi odnotowano ok. 20% zahamowanie tempa degradacji alachloru w trakcie pierwszych 24 h hodowli, natomiast w późniejszych etapach nie zaobserwowano różnic pomiędzy badanym układem a układem kontrolnym. Zastosowanie wyższych stężeń miedzi - 2,5 oraz 5 mM spowodowało silne zahamowanie biotransformacji ksenobiotyku (60-62%) w 24 h, natomiast ostatecznie spowodowały jedynie 10-13% pogorszenie degradacji badanego związku.

Wyniki badań dotyczących udziału enzymów oksydoredukcyjnych (lakazy, cytochromu P450) w eliminacji alachloru przez *T. koningii* IM 0956, a także oceny zdolności tego szczepu do jednoczesnej eliminacji herbicydu i jonów miedzi opublikowano w pracy eksperymetalnej "Potential of *Trichoderma koningii* to eliminate alachlor in the presence of copper ions" (Ecotoxicology and Environmental Safety 2018, 162, 1-9; 10.1016/j.ecoenv.2018.06.060 IF= 3.974, 30 pkt MNiSW).

Stwierdzenia cząstkowe dotyczące oceny zdolności wybranych szczepów z rodzaju *Trichoderma* do biotransformacji alachloru i metolachloru

1. Szczepy *Trichoderma: T. atroviride* IM QF10, *T. hamatum* IM I-1, *T. harzianum* IM 0961, *T. koningii* IM 0956, *T. citrinoviride* IM 6325, *T. harzianum* KKP 534, *T. viride* KKP 792 oraz *T. virens* DSM 1963 charakteryzują się zdolnością do eliminacji alachloru i metolachloru, ale różnią się pod względem efektywności degradacji tych związków.
2. Analiza widm masowych uzyskanych poprzez zastosowanie wysokosprawnej chromatografii cieczowej sprzężonej z tandemową spektrometrią mas umożliwiła identyfikację 8 metabolitów alachloru oraz 4 metabolitów metolachloru. Dwa

metabolity metolachloru: N-(2-etylo-6-metylofenylo)-2-hydroksy-N-(2-hydroksy-1-metyloetylo)acetamid oraz N-(2-etylo-6-metylofenylo)-N-(2-hydroksy-1-metyloetylo)acetamid zostały po raz pierwszy zidentyfikowane w hodowli mikroskopowych grzybów strzępkowych jako produkt degradacji tego herbicydu. Biotransformacja badanych ksenobiotyków przez *Trichoderma* spp. zachodzi głównie na drodze reakcji hydroksylacji, dechlorynacji oraz demetylacji.

3. Mikrobiologiczna transformacja alachloru i metolachloru prowadzi do zmniejszenia toksyczności środowiska, a w przypadku przekształceń alachloru przez szczepy *T. hamatum* IM I-1, *T. koningii* IM 0956 i *T. harzianum* IM 0956 stwierdzono niemal całkowitą detoksykację.

4. W biotransformacji alachloru przez szczep *T. koningii* IM 0956 biorą udział cytochrom P450 (szczególnie w początkowym etapie procesu) oraz lakaza. Proces zachodzi również w obecności jonów miedzi. Stężenia metalu w zakresie 2,5 - 5 mM powodują zahamowanie eliminacji jedynie o 10-13% .

III.2 Ocena wpływu wybranych szczepów *Trichoderma* na wzrost siewek rzepaku poddanych ekspozycji na badane herbicydy chloroacetanilidowe.

Aplikacja herbicydów o działaniu selektywnym, w tym także herbicydów chloroacetanilidowych oprócz niszczenia roślin docelowych może również działać niekorzystnie na uprawy roślin objętych przez nie ochroną (Ahemad i Kibret, 2014). Grzyby strzępkowe z rodzaju *Trichoderma* są kosmopolitycznymi mikroorganizmami o potwierdzonym pozytywnym wpływie na wzrost i rozwój roślin (Viterbo i in., 2010; Zhang i in., 2016). W związku z tym realizację drugiego celu niniejszej rozprawy doktorskiej rozpoczęto od oceny wpływu wybranych szczepów *Trichoderma* na długość pędów i korzeni 7-dniowych siewek rzepaku poddanych ekspozycji na alachlor (1,5 mg/L) i metolachlor (1,0 mg/L). Jako model roślinny wybrano rzepak, ponieważ jest jedną z najważniejszych i najpopularniejszych roślin oleistych uprawianych na całym świecie, a w ochronie jego upraw przed chwastami stosowane są herbicydy chloroacetanilidowe (Stamper i Tuovinen, 1998; Lin i in., 2013). Wykazano, iż w obecności szczepów *T. harzianum* KKP 534 oraz *T. viride* KKP 792 zwiększyła się długość korzeni i pędów siewek rzepaku odpowiednio o 38 i 45% w porównaniu do układu kontrolnego bez *Trichoderma* spp.. Stymulację wzrostu rzepaku poprzez aplikację zarodników badanych szczepów *Trichoderma* odnotowano zwłaszcza w obecności alachloru i metolachloru. W przypadku *T. koningii* IM 0956, *T. harzianum* KKP 534 oraz *T. viride* KKP 792 odnotowano pozytywny wpływ na wzrost korzeni. Natomiast inokulacja siewek rzepaku zarodnikami IM 0956, KKP 534, KKP 792 oraz DSM 1963 znacząco wpłynęła na wydłużenie pędów rzepaku. W pracy określono również poziom chlorofilu. W sadzonkach rzepaku narażonych na działanie metolachloru zaobserwowano statystycznie istotny wzrost zawartości chlorofilu w wyniku aplikacji pięciu spośród ośmiu badanych szczepów *Trichoderma*: *T. hamatum* IM I-1, *T. harzianum* IM 0961, *T. koningii* IM 0956, *T. citrinoviride* IM 6325 oraz *T. harzianum* KKP 534. Natomiast w przypadku oddziaływania alachloru pozytywny wpływ na poziom chlorofilu odnotowano podczas zastosowania zarodników IM 0961 oraz IM 0956.

W kolejnym etapie pracy zbadano zdolności grzybów do rozpuszczania fosforanów, wytwarzania sideroforów oraz produkcji deaminazy 1-aminocyklopropano-1-karboksylowej (ACCD). Aktywności te mogą świadczyć o promowaniu wzrostu roślin przez badane szczepy *Trichoderma*. Biodostępność fosforu, podstawowego makroskładnika odżywczego roślin,

w glebie jest ograniczona ze względu na występowanie w postaci nierozpuszczalnych form m.in. fosforanu wapnia, żelaza czy glinu. Źródła literaturowe wskazują, że grzyby z rodzaju *Trichoderma* sp. są zdolne do rozpuszczania fosforanów dzięki wytwarzaniu słabych kwasów organicznych (Saravanakumar i in., 2013). Uzyskane wyniki wskazują, że począwszy od pierwszych 24 godzin hodowli wszystkie badane szczepy grzybowe były zdolne do rozpuszczania fosforanów. Ich stężenie w supernatantach z płynnych hodowli szczepów *Trichoderma* wahało się od 132 do 164 mg/L, natomiast w kontrolach abiotycznych wynosiło 13-18 mg/L. Negatywny wpływ herbicydów na rozpuszczalność fosforanów odnotowano przez cały okres trwania hodowli *T. harzianum* KKP 534, *T. viride* KKP 792 oraz *T. virens* DSM 1963. Solubilizacja fosforanów przez wyżej wymienione szczepy była hamowana najintensywniej w 24 h inkubacji o odpowiednio 85, 50 i 65% w hodowlach z dodatkiem alachloru oraz 86, 53 i 58% w hodowlach z dodatkiem metolchloru. Natomiast dla pozostałych szczepów nie odnotowano znaczącego pogorszenia rozpuszczania fosforanów. Grzyby z rodzaju *Trichoderma* są również znane jako producenci sideroforów, metabolitów wtórnych wykazujących korzystne biologiczne działanie na wzrost roślin (Kumar Ghosh i in., 2015). Potwierdzono, że wszystkie badane szczepy charakteryzowały się zdolnością do wytwarzania sideroforów zarówno w układzie bez jak i z dodatkiem chloroacetanilidów. W hodowlach *Trichoderma* sp. z dodatkiem herbicydów odnotowano inhibicję wytwarzania sideroforów w ciągu pierwszych 24 h inkubacji. W starszych hodowlach chloroacetanilidy nie hamowały już syntezy sideroforów, a nawet w przypadku szczepu IM 0961 oraz KKP 792 zaobserwowano indukcję wytwarzania tych metabolitów wtórnych w obecności alachloru i metolachloru.

Dla szczepów *T. harzianum* IM 0961, *T. harzianum* KKP 534, *T. koningii* IM 0956 oraz *T. citrinoviride* IM 6325, które charakteryzowały się najlepszymi właściwościami promującymi wzrost roślin w obecności badanych chloroacetanilidów, a także największą efektywnością w ich biotransformacji, określono również aktywność deaminazy ACC (ACCD). ACCD jest enzymem hydrolizującym prekursor etylenu, a przez to bierze udział w obniżeniu jego poziomu w tkankach roślinnych, co prowadzi do opóźnienia starzenia się roślin oraz stymulacji odporności na różne czynniki stresowe (Viterbo i in., 2010). Aktywność deaminazy ACC stwierdzono u wszystkich wybranych szczepów *Trichoderma*. Największą aktywnością tego enzymu charakteryzował się szczep IM 0961 (52,14 µM α-ketomaślanu/mg białka/h)

czyli od 3 do 6-razy wyższą w porównaniu z pozostałymi szczepami. Ponadto wyniki wskazują również na indukcję aktywności enzymu w hodowli IM 0961 z dodatkiem alachloru i metolachloru. Mikroorganizmy promujące wzrost roślin w warunkach optymalnych mają aktywność ACCD na stosunkowo niskim poziomie, ale pod wpływem stresu biotycznego lub abiotycznego jej aktywność może być stymulowana, a tym samym może minimalizować działanie czynnika stresowego (Glick, 2014).

Wyniki badań dotyczących wybranych szczepów *Trichoderma* na wzrost siewek rzepaku poddanych ekspozycji na działanie alachloru i metolachloru oraz rezultaty eksperymentów mających na celu potwierdzenie potencjału badanych *Trichoderma* spp. w promowaniu wzrostu roślin zostały opublikowane w pracy doświadczalnej "Biotransformation and detoxification of chloroacetanilide herbicides by *Trichoderma* spp. with plant growth-promoting activities" (Pesticide Biochemistry and Physiology, 10.1016/j.pestbp.2019.11.018; IF= 344, 100 pkt MNiSW).

Stwierdzenia cząstkowe dotyczące oceny wpływu wybranych szczepów *Trichoderma* na wzrost siewek rzepaku poddanych ekspozycji na badane herbicydy chloroacetanilidowe.

1. Spośród badanych szczepów *Trichoderma*: *T. harzianum* KKP 534, *T. viride* KKP 792, *T. koningii* IM 0956 oraz *T. virens* DSM 1963 stymulują wzrost korzeni i pędów rzepaku zarówno w układzie z jak i bez dodatku alachloru i metolachloru.
2. Wszystkie szczepy *Trichoderma* wykazują cechy świadczące o wspomaganiu wzrostu roślin poprzez wytwarzanie sideroforów oraz rozpuszczanie fosforanów. Aktywności te występowały przez cały okres trwania hodowli. Nie wykazano negatywnego wpływ badanych chloroacetanilidów na proces wytwarzania związków chelatujących jony żelaza dla hodowli stacjonarnych. Natomiast w hodowlach 24 h prowadzonych w obecności herbicydów odnotowano wyraźne zahamowanie wytwarzania sideroforów. Dodatek herbicydów powodował pogorszenie rozpuszczania fosforanów przez cały czas inkubacji w przypadku trzech badanych szczepów: *T. harzianum* KKP 534, *T. viride* KKP 792 oraz *T. virens* DSM 1963.
3. Dla najbardziej aktywnych w degradacji szczepów wykazano aktywność deaminazy 1-aminocyklopropano-1-karboksylowej (ACC), enzymu, którego aktywność może

stymulować odporności na różne czynniki stresowe. Co istotne dla szczepu *T. harzianum* IM 0961 aktywność tego enzymu była stymulowana przez chloroacetanilidy.

III.3 Badanie oddziaływania herbicydów na szczepy *Trichoderma* zdolne do biotransformacji oraz analiza zmian adaptacyjnych indukowanych obecnością tych związków.

Jednym z mechanizmów toksycznego oddziaływania alachloru i metolachloru jest indukcja stresu oksydacyjnego poprzez nadprodukcję reaktywnych form tlenu (RFT), co w rezultacie może prowadzić do uszkodzenia biomolekuł (Jang i in., 2016). Dotychczas prowadzone badania dotyczące stresu oksydacyjnego oraz jego konsekwencji dotyczyły głównie roślin (Singh i in., 2017; Štajner i in., 2004), kręgowców (Yi i in., 2007), a także tkanek zwierzęcych (Burman i in., 2003). Niemniej jednak wciąż niewiele wiadomo na temat odpowiedzi na stres oksydacyjny mikroorganizmów zdolnych do efektywnej eliminacji chloroacetanilidów. W związku z tym realizację ostatniego celu niniejszej rozprawy doktorskiej rozpoczęto od oceny indukcji stresu oksydacyjnego w hodowlach badanych grzybów mikroskopowych poddanych ekspozycji na alachlor i metolachlor poprzez zastosowanie popularnych biomarkerów. W pierwszym etapie przeprowadzono detekcję *in situ* anionorodnika ponadtlenkowego ($O_2^{\bullet-}$) oraz rodnika hydroksylowego/nadtlenoazotynu ($HO\bullet/ONOO^-$) w grzybniach ośmiu szczepów *Trichoderma*: *T. atroviride* IM QF10, *T. hamatum* IM I-1, *T. harzianum* IM 0961, *T. koningii* IM 0956, *T. citrinoviride* IM 6325, *T. harzianum* KKP 534 oraz *T. virens* DSM 1963 poddanych ekspozycji na działanie alachloru i metolachloru. Detekcja wyżej wymienionych RFT została wykonana poprzez zastosowanie barwników, służących do oceny komórkowego stresu oksydacyjnego - błękitu nitrotetrazoliowego (NBT) dla anionorodnika ponadtlenkowego ($O_2^{\bullet-}$) oraz dioctanu 2',7'-dichlorodihydrofluoresceiny (H_2DCFDA) dla rodnika hydroksylowego/nadtlenoazotynu ($HO\bullet/ONOO^-$). H_2DCFDA jest niespecyficznym wskaźnikiem stresu oksydacyjnego i jest powszechnie stosowany do określania ogólnego poziomu reaktywnych form tlenu (Słaba i in., 2015; Bernat i in., 2018; Maronić i in., 2018). Jednakże według Kalyanaraman i wsp. (2012), a także informacji zawartych w Molecular Probes Handbook (Termofisher) (Johnson i Spence, 2010) barwnik ten należy stosować dla określenia poziomu HO• jak i $ONOO^-$. Uzyskane w pracy wyniki wskazują na wysoki poziom wewnątrzkomórkowego $O_2^{\bullet-}$, a jednocześnie dużo niższy poziom $HO\bullet/ONOO^-$, a zatem stosując barwnik H_2DCFDA i wykrywając tylko $HO\bullet/ONOO^-$ nie zawsze można określić ogólną pulę RFT. Najwyższy poziom reaktywnych form tlenu odnotowano w hodowlach *Trichoderma* spp. pochodzącym z wykładniczej fazy wzrostu (24 h). Zaobserwowano, iż chloracetanilidy wpływały znacząco

na nadprodukcję anionorodnika ponadtlenkowego w porównaniu do poziomu drugiego wykrywanego rodnika HO•/$ONOO^-$. Obecność chloroacetanilidów spowodowała 4-8-krotny wzrost poziomu $O_2^{\bullet-}$ w hodowlach IM 0956, IM QF10, IM 0961 i IM 6325, natomiast w hodowlach KKP 534, KKP 792 oraz DSM 1963 odnotowano 15-28-krotny wzrost anionorodnika ponadtlenkowego. Odnotowano również zwiększone wytwarzanie rodnika hydroksylowego/nadtlenoazotynu w grzybni *Trichoderma* spp. w wyniku oddziaływania herbicydów, jednakże w większości przypadków bez statystycznego znaczenia. Uzyskane wyniki wskazują na indukcję stresu oksydacyjnego w wykładniczej fazie wzrostu u większości badanych *Trichoderma* spp.. Natomiast w stacjonarnej fazie wzrostu (72 h) zaobserwowano istotny spadek poziomu zarówno $O_2^{\bullet-}$ jak i HO•/$ONOO^-$, co wskazuje na aktywną odpowiedź systemu antyoksydacyjnego. Indukcja RFT oraz ich wewnątrzkomórkowa akumulacja może być również spowodowana wysoką aktywnością metabolizmu szczepów *Trichoderma*, podczas biotransformacji badanych chloroacetanilidów.

Ponieważ w pierwszym etapie realizacji tego celu pracy wykazano wzmożony poziom RFT, sprawdzono również czy alachlor i metolachlor wywołują oksydacyjne uszkodzenia lipidów oraz białek w hodowlach badanych grzybów strzępkowych. Peroksydację lipidów oceniono poprzez pomiar zawartości substancji reagujących z kwasem tiobarbiturowym (TBARS) oraz grup karbonylowych w przypadku utleniania białek. Zaobserwowano wzrost poziomu produktów peroksydacji lipidów w próbach z dodatkiem herbicydów zarówno w wykładniczej (24 h) jak i stacjonarnej (72 h) fazie wzrostu u siedmiu spośród ośmiu szczepów *Trichoderma* (1,5-3-krotny w porównaniu do układu kontrolnego). Jedynie w przypadku szczepu DSM 1963 nie odnotowano żadnych zmian w poziomie TBARS pod wpływem alachloru i metolachloru. Wyniki wskazują, że wzmożona nadprodukcja TBARS w ciągu pierwszych 24 h inkubacji jest spowodowana nadprodukcją RFT, a także potwierdzają indukcję stresu oksydacyjnego u badanych grzybów pod wpływem chloroacetanilidów. Natomiast akumulacja produktów peroksydacji lipidów w fazie stacjonarnej, kiedy dla większości badanych szczepów odnotowano niższy niż w 24 h poziom rodników, może być związany z łańcuchowym charakterem reakcji zaangażowanych w ten proces (Li i in., 2009). W wykładniczej fazie wzrostu odnotowano również wzrost poziomu produktów peroksydacji białek w hodowlach IM 6325, IM I-1 i KKP 792 z dodatkiem alachloru oraz w hodowlach IM QF 10, IM 6325, IM I-1, IM 0956 oraz DSM 1963 z dodatkiem

metolachloru. Podwyższony poziom grup karbonylowych także jest skorelowany z nadprodukcją RFT i jest kolejnym dowodem potwierdzającym indukcję stresu oksydacyjnego w hodowlach *Trichoderma* spp.. Z kolei po trzech dniach inkubacji w wyżej wymienionych hodowlach zaobserwowano spadek zawartości grup karbonylowych, co sugeruje aktywną odpowiedź systemu antyoksydacyjnego badanych szczepów. Kolejną możliwością jest zaangażowanie enzymów proteolitycznych w eliminację zmodyfikowanych oksydacyjnie białek, które często są określane mianem wtórnej obrony antyoksydacyjnej (Nyström, 2005)

Odpowiedź systemu antyoksydacyjnego szczepów *Trichoderma* została oceniona poprzez pomiar aktywności enzymów - katalazy (CAT) i dysmutazy ponadtlenkowej (SOD), które stanowią pierwszą linię obrony przeciwko stresowi oksydacyjnemu. Zaobserwowano zróżnicowaną odpowiedź badanych enzymów antyoksydacyjnych na stres wywołany obecnością alachloru i metolachloru w hodowlach grzybów strzępkowych z rodzaju *Trichoderma*. Po 3 dniach inkubacji odnotowano 2-6-krotną indukcję aktywności katalazy w hodowlach IM 6325, KKP 534, KKP 792 i DSM 1963 z dodatkiem ksenobiotyków w porównaniu do prób pochodzących z pierwszych 24 h hodowli, co wskazuje na aktywną odpowiedź systemu antyoksydacyjnego. Natomiast zwiększony poziom anionorodnika ponadtlenkowego w próbach z dodatkiem herbicydów nie znalazł odzwierciedlenia w indukcji aktywności dysmutazy ponadtlenkowej u większości badanych mikroorganizmów. Jedynie w przypadku hodowli IM I-1 i DSM 1963 z dodatkiem alachloru oraz KKP 792 z dodatkiem metolachloru odnotowano stymulację aktywności SOD po 24 godzinach inkubacji.

Mechanizm działania chloroacetanilidów, w tym także alachloru i metoalchloru opiera się na zahamowaniu syntezy białek i kwasów tłuszczowych o długich łańcuchach węglowych (Souissi i in., 2013). Zmiany w składzie lipidów błonowych, zwłaszcza fosfolipidów (PLs), odgrywają ważną rolę w adaptacji do różnych warunków stresowych (Bernat i in., 2018b; De Kroon i in., 2013; Słaba i in., 2013b; Stolarek i in., 2019; Zawadzka i in., 2017). W związku z tym realizację ostatniego celu pracy kontynuowano poddając analizie wpływ badanych chloracetanilidów na skład fosfolipidów błonowych ośmiu szczepów *Trichoderma*. Zidentyfikowano następujące klasy lipidów: fosfatydylocholiny (PC), fosfatydyloetanoloaminy (PE), kwas fosfatydowy (PA), fosfatydyloseryny (PS) oraz

fosfatydyloinozytol (PI). Dominującą klasą fosfolipidów były fosfatydylocholiny stanowiące 31,63-82,23% wszystkich analizowanych klas. Natomiast zawartość PE, PA, PI oraz PS w układach kontrolnych wynosiła odpowiednio 7,12–37,42, 0,41–2285, 0,71–12,48 oraz 0,31–6,59%. Spośród dwóch badanych herbicydów metolachlor wyraźnie modyfikował profil lipidowy *Trichoderma* spp. w stacjonarnej fazie wzrostu, co przejawiało się zmianami ilościowymi w składzie PA, PC oraz PE. W hodowlach IM QF10, IM I-1 oraz IM 0956 z dodatkiem metolachloru odnotowano wzrost zawartości PE, który jest skorelowany ze zmniejszoną zawartością PC, co sugeruje usztywnienie błony komórkowej badanych grzybów strzępkowych. Natomiast odwrotną korelację zaobserwowano w hodowlach IM 6325, IM 0961 i KKP 534 z dodatkiem metolachloru, a także w hodowlach IM 0961, IM I-1, IM 0956 oraz KKP 534 z dodatkiem alachloru, co z kolei wskazuje na wzrost płynności błony *Trichoderma* spp.. Ponadto w wyniku ekspozycji na metolachlor zaobserwowano również w fazie stacjonarnej wyraźny wzrost poziomu kwasu fosfatydowego u IM I-1, IM 0956 oraz KKP 534. PA jest lipidem sygnałowy, którego poziom w dwuwarstwie lipidowej może ulec zwiększeniu w wyniku oddziaływania stresu abiotycznego lub biotycznego (Testerink i Munnik, 2005). Działanie metolachloru potwierdza te doniesienia.

Kondycję błony komórkowej szczepów *Trichoderma* spp. narażonych na działanie alachloru i metolachloru określono poprzez pomiar wewnątrzkomórkowej akumulacji jodku propidu wewnątrz strzępek badanych drobnoustrojów. Obecność ksenobiotyków spowodowała minimalny wzrost przepuszczalności błony zarówno w wykładniczej jak i stacjonarnej fazie wzrostu, jednakże w większości przypadków bez znaczenia statycznego. Pomimo wzrostu poziomu produktów peroksydacji lipidów oraz zmian w składzie fosfolipidów, nie zaobserwowano znaczących zmian w przepuszczalności błon komórkowych *Trichoderma* spp. w wyniku ekspozycji na alachlor i metolachlor.

Wyniki badań dotyczące oddziaływania herbicydów na szczepy *Trichoderma* zdolne do biotransformacji oraz zmian adaptacyjnych w odpowiedzi na obecność tych związków zostały opublikowane w pracy oryginalnej "Assessment of oxidative stress and phospholipids alterations in chloroacetanilides-degrading *Trichoderma* spp.." (Ecotoxicology and Environmenral Safety 184; 10.1016/j.ecoenv.2019.109629, IF=3.974, 100 pkt MNiSW).

Stwierdzenia cząstkowe dotyczący oceny oddziaływania herbicydów na szczepy *Trichoderma* zdolne do biotransformacji oraz zmian adaptacyjnych w odpowiedzi na obecność tych związków.

1. Wzrost poziomu wewnątrzkomórkowych reaktywnych form tlenu (RFT), produktów peroksydacji lipidów (TBARS) oraz białek (grup karbonylowych) sugerują indukcję stresu oksydacyjnego w fazie wzrostu wykładniczego (24 h) badanych szczepów *Trichoderma* poddanych ekspozycji na działanie alachloru lub metolachloru.
2. Znaczący spadek RFT oraz produktów peroksydacji białek, a także zmiany w aktywności katalazy w fazie stacjonarnej (72 h) wskazują na aktywną odpowiedź systemu antyoksydacyjnego badanych grzybów strzępkowych w odpowiedzi na stres wywołany obecnością chloroacetanilidów.
3. Zmiany w profilu fosfolipidowym grzybów *Trichoderma* w odpowiedzi na toksyczne działanie herbicydów wskazują na mechanizm adaptacyjny, który może być związany z tolerancją *Trichoderma* spp. na badane związki.
4. Mechanizm toksycznego działania herbicydów chloroacetanilidowych nie jest związany ze zwiększeniem przepuszczalności błony komórkowej *Trichoderma* spp.

IV. Podsumowanie i wnioski końcowe

1. Badane szczepy *Trichoderma*: *T. atroviride* IM QF10, *T. hamatum* IM I-1, *T. harzianum* IM 0961, *T. koningii* IM 0956, *T. citrinoviride* IM 6325, *T. harzianum* KKP 534, *T. viride* KKP 792 oraz *T. virens* DSM 1963 charakteryzują się wysoką wydajnością eliminacji alachloru jak i metolachloru, a szczególnie alachloru, który jest szybciej i efektywniej usuwany z hodowli.

2. Biotransformacja ksenobiotyków przez *Trichoderma* spp. przebiega na drodze reakcji hydroksylacji, demetylacji oraz dechlorynacji. Zidentyfikowano 8 pochodnych alachloru i 4 pochodne metalochloru, w tym 2 po raz pierwszy zidentyfikowane jako produkty metabolizmu grzybów.

3. Biotransformacja alachloru i metolachloru przez szczepy *Trichoderma* prowadzi do zmniejszenia toksyczności środowiska, a w przypadku eliminacji alachloru przez szczepy *T. hamatum* IM I-1, *T. harzianum* IM 0961 oraz *T. koningii* IM 0956 stwierdzono niemal całkowitą detoksykację.

4. Proces przekształcania alachloru przez szczep *T. koningii* IM 0956 obejmuje udział cytochromu P450 oraz lakazy. Ponadto szczep IM 0956 jest zdolny do efektywnej eliminacji alachloru w obecności wysokich stężeń jonów miedzi.

5. Wybrane szczepy *Trichoderma* stymulują wzrost siewek rzepaku w układzie bez jak i z dodatkiem badanych herbicydów oraz minimalizują ich niekorzystny wpływ na rzepak. Wykazują również cechy świadczące o promowaniu wzrostu roślin, m.in. zdolność do wytwarzania sideroforów, rozpuszczanie fosforanów oraz aktywność deaminazy ACC.

6. Spadek zawartości wewnątrzkomórkowych reaktywnych form tlenu (RFT), produktów peroksydacji białek i wzmożona aktywność katalazy podczas biotransformacji chloroacetanilidów przez szczepy *Trichoderma* wskazuje na aktywną odpowiedź systemu antyoksydacyjnego oraz mechanizm adaptacji badanych drobnoustrojów.

7. Zmiany w ogólnej zawartości dwóch głównych klas fosfolipidów - fosfatydylocholin oraz fosfatydyloetanoloamin można również uznać za mechanizm adaptacyjny szczepów *Trichoderma* chroniący przed toksycznym oddziaływaniem chloroacetanilidów.

8. Spośród badanych szczepów *Trichoderma* najbardziej interesującym modelem badawczym jest *T. koningii* IM 0956 ze względu na jedną z najlepszych aktywności degradacyjnych herbicydów połączoną z jednoczesnym promowaniem wzrostu, również w obecności alachloru i metolachloru.

V. Streszczenie

Alachlor i metolachlor są przed- i powschodowymi herbicydami, powszechnie stosowanymi w ochronie upraw rolniczych i ogrodniczych ze względu na dużą skuteczność oraz umiarkowaną trwałość w środowisku. Z powodu niekontrolowanego oraz nadmiernego stosowania tych herbicydów, substancje te stanowią realne zagrożenie dla środowiska naturalnego oraz organizmów niebędących celem zwalczania. Biodegradacja jest najważniejszą i najefektywniejszą drogą eliminacji chloroacetanilidów ze środowiska, a kluczową rolę w tym procesie przypisuje się mikroorganizmom wyizolowanym ze skażonych środowisk, które często charakteryzują się tolerancją w stosunku do toksycznych związków.

Niniejsza praca doktorska dotyczy zdolności wybranych szczepów *Trichoderma* w biotransformacji alachloru i metolachloru z jednoczesnym uwzględnieniem oddziaływania tychże związków na system antyoksydacyjny oraz profil lipidowy modelu badawczego. Ponadto poddano również ocenie zdolność *Trichoderma* spp. do promowania wzrostu roślin.

W trakcie realizacji pierwszego celu rozprawy doktorskiej wykazano, iż wszystkie badane szczepy *Trichoderma*: *T. atroviride* IM QF10, *T. hamatum* IM I-1, *T. harzianum* IM 0961, *T. koningii* IM 0956, *T. citrinoviride* IM 6325, *T. harzianum* KKP 534, *T. viride* KKP 792 oraz *T. virens* DSM 1963 charakteryzują się zdolnością do eliminacji alachloru i metolachloru, ale różnią się pod względem efektywności degradacji tych związków. Metabolizm chloroacetanilidów przez badane grzyby strzępkowe przebiega głównie na drodze reakcji dechlorynacji, demetylacji oraz hydroksylacji. Analiza toksyczności produktów mikrobiologicznej degradacji ksenobiotyków wskazuje na zmniejszenie toksyczności środowiska. Stwierdzono również, że w przypadku szczepu *T. koningii* IM 0956 eliminacja alachloru jest złożonym procesem obejmującym udział zarówno cytochromu P450 jak i lakazy. Kolejny cel naukowy dotyczył wpływu badanych szczepów *Trichoderma* na wzrost 7-dniowych siewek rzepaku poddanych ekspozycji na alachlor i metolachlor. Spośród badanych szczepów *Trichoderma*: *T. harzianum* KKP 534, *T. viride* KKP 792, *T. koningii* IM 0956 oraz *T. virens* DSM 1963 stymulowały wzrost korzeni i pędów rzepaku zarówno w układzie z jak i bez dodatku herbicydów. Wykazano również, że wszystkie szczepy wykazują cechy świadczące o promowaniu wzrostu m.in. zdolność do wytwarzania sideroforów czy rozpuszczanie fosforanów. Dla najbardziej aktywnych w degradacji

Trichoderma spp. wykazano aktywność deaminazy 1-aminocyklopropano-1-karboksylowej (ACC), a dla szczepu *T. harzianum* IM 0961 aktywność tego enzymu była wyższa po ekspozycji na chloroacetanilidy. Podczas realizacji ostatniego celu niniejszej pracy sprawdzono oddziaływania alachloru i metolachloru na drobnoustroje zdolne do ich efektywnej biotransformacji. Detekcja *in situ* wewnątrzkomórkowych reaktywnych form tlenu oraz obecność produktów peroksydacji białek i lipidów w wykładniczej fazie wzrostu badanych *Trichoderma* spp. potwierdziła indukcję stresu oksydacyjnego w wyniku ekspozycji na alachlor i metolachlor. Z drugiej strony znaczący spadek RFT oraz produktów peroksydacji białek, a także zmiany w aktywności katalazy w fazie stacjonarnej (72 h) wskazują na aktywną obronę systemu antyoksydacyjnego badanych grzybów strzępkowych w odpowiedzi na stres wywołany obecnością chloroacetanilidów. Zmiany w profilu fosfolipidowym grzybów *Trichoderma,* zwłaszcza w obrębie dwóch głównych klas PC i PE w odpowiedzi na toksyczne działanie herbicydów, mogą świadczyć o mechanizmie adaptacyjnym. Ponadto nie odnotowano statystycznie znaczącego wzrostu przepuszczalności błon komórkowych *Trichoderma* spp po ekspozycji na chloroacetanilidy.

Badania przeprowadzone w trakcie tworzenia niniejszej rozprawy doktorskiej mają charakter podstawowy, niemniej jednak wykazały możliwość potencjalnego zastosowania kosmopolitycznych grzybów strzępkowych z rodzaju *Trichoderma* w bioremediacji obszarów rolniczych skażonych pozostałościami chloroacetanilidów. Jest to szczególnie istotne w przypadku alachloru, którego stosowanie w UE zostało zabronione m.in. ze względu na ksenoestrogenne właściwości. Spośród badanych szczepów *Trichoderma* najbardziej interesującym modelem badawczym jest *T. koningii* IM 0956 ze względu na jedną z najlepszych aktywności degradacyjnych herbicydów połączoną z jednoczesnym promowaniem wzrostu, również w obecności alachloru i metolachloru.

VI. Abstract

Alachlor and metolachlor are pre- and post-emergent herbicides commonly used in the protection of agricultural and horticultural crops due to their high effectiveness and moderate environmental stability. Owing to the uncontrolled and excessive use of these herbicides, they pose a real threat to the natural environment and non-target organisms. Biodegradation is the most important method of controlling the dissipation of chloroacetanilide herbicides in the natural environment, a key role in this process is attributed to microorganisms isolated from contaminated environments, which are often characterized by tolerance to toxic compounds.

This doctoral thesis focuses on the analysis of the ability of selected *Trichoderma* strains to biotransform alachlor and metolachlor with a simultaneous consideration of the impact of these compounds on the antioxidant system and lipid profile of the fungal research model. Moreover, *Trichoderma* spp. ability to promote plant growth has been evaluated.

During the study, it was shown that all tested *Trichoderma* strains: *T. atroviride* IM QF10, *T. hamatum* IM I-1, *T. harzianum* IM 0961, *T. koningii* IM 0956, *T. citrinoviride* IM 6325, *T. harzianum* KKP 534, *T viride* KKP 792 and *T. virens* DSM 1963 were characterized by the ability to eliminate alachlor and metolachlor but differed in the efficiency of degradation of the xenobiotics. Biotransformation of the herbicides was performed mainly by the reaction of dechlorination, demethylation and hydroxylation. The analysis of the toxicity of microbial degradation products of chloroacetanilides indicated a reduction in environmental toxicity. It was also found that in the case of the strain *T. koningii* IM 0956, the elimination of alachlor was a complex process involving both cytochrome P450 and laccase. The subsequent aim of the thesis concerned the impact of the studied *Trichoderma* strains on the growth of 7-day rape seedlings exposed to alachlor and metolachlor. Among the tested *Trichoderma* strains, *T. harzianum* KKP 534, *T. viride* KKP 792, *T. koningii* IM 0956 and *T. virens* DSM 1963 stimulated the growth of roots and shoots of rapeseed seedlings treated with alachlor or metolachlor. All tested strains exhibited plant growth-promoting traits, such as siderophore production and phosphate solubilization, even in the presence of chloroacetanilide herbicides. *Trichoderma* spp. with the highest capability to effectively eliminated tested chloroacetanilides, were also characterized by the ability to produce

1-aminocyclopropane-1-carboxylic deaminase (ACCD), and for the *T. harzianum* IM 0961 strain the activity of this enzyme was higher after exposure to chloroacetanilides. The last stage of the thesis was an assessment of the effect of alachlor and metolachlor on microorganisms capable of their effective biotransformation. The overproduction of intracellular reactive oxygen species (especially the superoxide anion) and increased lipid and protein oxidative damage confirmed chloroacetanilide-induced stress in *Trichoderma* spp. cultures during the exponential phase of growth. In turn, a considerable decline in the ROS levels and the carbonyl group content (biomarkers of protein peroxidation) in a time-dependent manner and changes in the antioxidant enzyme activities indicated an active response against chloroacetanilide-induced oxidative stress and the mechanism of tolerance in the tested fungi. The changes in the overall content of two main PLs groups (PC and PE) can be considered as defense response of fungal cells against the toxic action of chloroacetanilides. In addition, there was no statistically significant increase observed in the permeability of *Trichoderma* spp. cell membranes after exposure to chloroacetanilides.

The research carried out during the creation of this doctoral dissertation has a basic character, however, it has shown the potential for the use of cosmopolitan filamentous fungi from the genus *Trichoderma* in bioremediation of agricultural areas contaminated with chloroacetanilides. Among the tested *Trichoderma* strains, *T. koningii* IM 0956 has been found to be the most interesting research model because of its the high ability to eliminate the tested chloroacetanilides and multiple PGP activities even in the presence of the herbicides.

VII. Literatura

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Całkowity dorobek naukowy

Publikacje

1. Słaba M., Bernat P., Różalska S., **Nykiel J**., Długoński J. 2013. Comparative study of metal induced phospholipid modifications in the heavy metal tolerant filamentous fungus *Paecilomyces marquandii* and implications for the fungal membrane integrity. Acta Biochim. Pol. 60: 695-700.

   IF= 1.389, MNiSW = 15

2. **Nykiel-Szymańska J**., Stolarek P., Bernat P. 2017. Elimination and detoxification of 2,4-D by *Umbelopsis isabellina* with the involvement of cytochrome P450. Environ. Sci. Pollut. Res. Int. 25:2738-2743.

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3. Bernat P., **Nykiel-Szymańska J**., Stolarek P., Słaba M., Szewczyk R., Różalska S. 2018. 2,4-dichlorophenoxyacetic acid-induced oxidative stress: Metabolome and membrane modifications in *Umbelopsis isabellina,* a herbicide degrader. PLoS ONE 13(6): e0199677.

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4. **Nykiel-Szymańska J**., Bernat P., Słaba M. 2018. Potential of *Trichoderma koningii* to eliminate alachlor in the presence of copper ions. Ecotoxicol. Environ. Saf. 162:1-9.

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5. Bernat P., **Nykiel-Szymańska J**., Gajewska E., Różalska S., Stolarek P., Dackowa J., Słaba M. 2018. *Trichoderma harzianum* diminished oxidative stress caused by dichlorophenoxyacetic acid (2,4-D) in wheat, with insights from lipidomics. J. Plant. Physiol. 229:158-163.

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6. **Nykiel-Szymańska J.**, Bernat P., Słaba M. (w druku) Biotransformation and detoxification of chloroacetanilide herbicides by *Trichoderma* spp. with plant growth-promoting activities. Pestic. Biochem. Physiol., 10.1016/j.pestbp.2019.11.018

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7. **Nykiel-Szymańska J.**, Różalska S., Bernat P., Słaba M. **2019**. Assessment of oxidative stress and phospholipids alterations in chloroacetanilides-degrading *Trichoderma* spp.. Ecotoxicol. Environ. Saf. 184, 10.1016/j.ecoenv.2019.109629

IF= 3.974, MNiSW = 100

Sumaryczny IF= 21.176

Łączna liczba punktów MNiSW=345

Liczba cytowań=26

Index H=3

Wartości IF oraz punktację MNiSW podano zgodnie z rokiem opublikowania z wyjątkiem publikacji nr 6, dla której przyjęto wartości IF oraz punktów MNiSW z 2019 r.

Komunikaty zjazdowe

a) prezentacje ustne

1. **Nykiel J.**, Siewiera P., Słaba M., Długoński J. 2014. Wpływ alachloru na przepuszczalność błony komórkowej i system antyoksydacyjny grzyba strzępkowego *Paecilomyces marquandii.* Mała Wielka Nauka. Mikro, nano i co dalej?
2. Siewiera P., **Nykiel J.**, Długoński J. 2014. Mikrobiologiczna degradacja związków cyny. Mała Wielka Nauka. Mikro, nano i co dalej?
3. **Nykiel J.**, Siewiera P., Słaba M., Długoński J. 2015. Odpowiedź systemu antyoksydacyjnego grzybów strzępkowych z rodzaju *Trichoderma* na stres wywołany obecnością alachloru. PUZZEL 2015, IV Wrocławska Konferencja Studentów Nauk Technicznych i Ścisłych.
4. Siewiera P., **Nykiel J.**, Długoński J. 2015. Wpływ tributylocyny (TBT) na skład fosfolipidowy i przepuszczalność błon komórkowych u wybranych grzybów mikroskopowych. PUZZEL 2015, IV Wrocławska Konferencja Studentów Nauk Technicznych i Ścisłych.
5. **Nykiel J.**, Siewiera P., Słaba M. 2015. Eliminacja alachloru przez mikroskopowy grzyb strzępkowy *Trichoderma atroviride*. Mała Wielka Nauka. Innowacje i idee przyszłości.
6. **Nykiel J.**, Siewiera P., Słaba M. 2015. Eliminacja alachloru przez mikroskopowe grzyby strzępkowe. PUZZEL 2016. V Wrocławska Konferencja Studentów Nauk Technicznych i Ścisłych.
7. **Nykiel J.**, Siewiera P., Różalska S., Słaba M. 2015. Detection reactive oxygen species and elevation of antioxidant enzymes activity in microscopic filamentous fungi exposed to alachlor. VII International Conference of Biotechnology Students & XVII National Academic Seminar of Biotechnology Students.
8. Siewiera P., **Nykiel J.**, Bernat P. 2016. Biodegradacja związków cynoorganicznych przez wybrane szczepy bakteryjne i grzybowe z uwzględnieniem roli cytochromu P-450. V Wrocławska Konferencja Studentów Nauk Technicznych i Ścisłych.
9. **Nykiel J.,** Siewiera P., Bernat P., Słaba M. 2016. Eliminacja kwasu 2,4- dichloroctowego przez grzyby strzępkowe z rodzaju *Fusarium.* Mała Wielka Nauka. W labiryncie nauki.
10. Siewiera P., **Nykiel J.,** Bernat P. 2016. Rola przeciwutleniaczy w procesie eliminacji związków o właściwościach prooksydacyjnych. Mała Wielka Nauka. W labiryncie nauki.

11. Siewiera P., **Nykiel-Szymańska J**., Bernat P.2017. Oddziaływanie tributylocyny (TBT) na profil fosfolipidowy, skład kwasów tłuszczowych i przepuszczalność błon komórkowych wybranych grzybów mikroskopowych. PUZZEL 2017. VI Wrocławska Konferencja Studentów Nauk Technicznych i Ścisłych.

12. Nykiel-Szymańska J., Siewiera P., Słaba M. 2017. Detekcja reaktywnych form tlenu oraz pomiar aktywności enzymów antyoksydacyjnych u wybranych szczepów *Trichoderma* poddanych działaniu alachloru. PUZZEL 2017. VI Wrocławska Konferencja Studentów Nauk Technicznych i Ścisłych.

13. Nykiel-Szymańska J., Siewiera P., Słaba M. 2017. Wpływ miedzi na eliminację alachloru przez mikroskopowy grzyb strzępkowy *Trichoderma koningii* I Sesja Mikrobiologów Środowiska Łódzkiego.

14. Bernat P., **Nykiel-Szymańska J.**, Siewiera P., Dackowa J., Pietrzak M. 2017. Lipidomic adaptations in the filamentous fungus, auxin herbicides. 6th Central European Congress of Life Science EUROBIOTECH.

15. Bernat P., **Nykiel-Szymańska J.**, Siewiera P., Dackowa J., Pietrzak M. 2017. Changes in lipidome composition of Mucorales treated with toxic lipophilic compounds. 4th Workshop on Microbiology in Health and Environmental Protection MIKROBIOT 2017.

16. Nykiel-Szymańska J., Stolarek P., Różalska S., Słaba M. 2017. Detekcja markerów stresu oksydacyjnego podczas eliminacji metolachloru przez wybrane szczepy *Trichoderma.* Mała Wielka Nauka IV Edycja: Pasja, Wiedza, Nauka.

17. Stolarek P., **Nykiel-Szymańska** J., Bernat P. 2017. Analiza jakosciowa i ilosciowa sfingolipidów grzybni *Meterhizium robertsii* poddanej ekspozycji na dibutylocynę (DBT). MAŁA WIELKA NAUKA IV Edycja: Pasja, Wiedza, Nauka.

18. Nykiel-Szymańska J., Stolarek P., Bernat P., Słaba M. 2018. Aktywność systemu antyoksydacyjnego mikroskopowych grzybów strzępkowych podczas eliminacji ksenobiotyków. Nowe Wyzwania dla Polskiej Nauki II Edycja.

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Biotransformation and detoxification of chloroacetanilide herbicides by *Trichoderma* spp. with plant growth-promoting activities

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ABSTRACT

Due to the increasing use of chlorinated organic compounds, environmental pollution is a key issue in agricultural and industrial areas. In this study, biodegradation of chloroacetanilide herbicides, such as alachlor and metolachlor, by eight fungal strains of *Trichoderma* spp. originating from different microorganism collections was investigated. The tested fungi converted 80–99% of alachlor and 40–79% of metolachlor after 7 days of incubation. Biotransformation of herbicides was performed mainly by dechlorination and hydroxylation reactions. Eight alachlor metabolites and four byproducts of metolachlor conversion were detected in *Trichoderma* cultures, including two metolachlor intermediates for the first time identified in fungi. Moreover, in the cultures of six *Trichoderma* strains supplemented with chloroacetanilides, a decrease in toxicity was observed toward tested *Artemia franciscana* crustaceans. Simultaneously, 7 days after the application of the spores of *T. koningii* IM 0956, *T. citrinoviride* IM 6325, *T. harzianum* KKP 534, *T. viride* KKP 792 and *T. virens* DSM 1963 the length of roots and shoots of rapeseed seedlings treated with alachlor or metolachlor significantly increased. All tested strains exhibited plant growth-promoting traits, such as siderophore production, 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity, and phosphate solubilization, even in the presence of chloroacetanilide herbicides.

1. Introduction

The excessive use of plant protection products, pesticides and industrial process products has led to the widespread contamination of the environment by organic pollutants. Currently, pesticide consumption in the world is approximately 2.5 million tons, of which herbicides account for > 40% (Fenner et al., 2013). Only a minimum fraction of the applied pesticide goes to the target organism (0.1%), while the rest is dispersed in the environment contaminating soil, surface water and groundwater, thus posing a threat to living organisms (Olchanheski et al., 2014). Alachlor [2-chloro-N-2,6-diethylphenyl-N-(methoxymethyl)acetamide] and metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide] are chloroacetanilide herbicides used widely to control annual grasses and broadleaf weeds in corn, cotton, soybean, rapeseed, rice, sunflower, and vegetables. Compounds belonging to this group of herbicides possess good water solubility and a low degree of mineralization, which facilitates the infiltration into groundwater and surface water (Huang et al., 2017). This is particularly dangerous because chloroacetanilides can exert harmful effects on living organisms. These herbicides are highly toxic to aquatic organisms. Alachlor belongs to the class of possible human carcinogens, whereas metolachlor is not classifiable to human carcinogenicity (United States EPA, 1995, 1998). Moreover, alachlor has been classified as an endocrine-disrupting compound (Bergman et al., 2012). Due to this reason, it has been banned in the European Union (EU) since 2008. However, metolachlor is still approved for use in EU countries. Biodegradation is the most important method to control the dissipation of chloroacetanilide herbicides in the natural environment (Huang et al., 2017). Numerous studies have been devoted to the use of soil autochthonous microorganisms to eliminate chloroacetamides, while a limited number of studies have been focused on the mechanism of the degradation process, identification of metabolites, and checking detoxification (Durães Sette et al., 2004; Munoz et al., 2011; Pothuluri et al., 1993, 1997; Sanyal and Kulshrestha, 2003; Słaba et al., 2013, 2015).

Trichoderma spp. are well-studied cosmopolitan fungi that inhibit the growth of phytopathogens, exert beneficial effect on plant growth, possess high enzymatic activity (cellulase, chitinase, pectinase, oxidoreductase), and offer resistance to a wide range of toxicants such as heavy metals (e.g., Cd, Cu, and Ni), agrochemicals (e.g.,

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dichlorodiphenyltrichloroethane, dieldrin, and chlorpyrifos), and harmful chemicals (e.g., polycyclic aromatic hydrocarbons (PAHs) and cyanide) (Blaya et al., 2013; Tripathi et al., 2013). Xenobiotics degradation by *Trichoderma* spp. has been well documented (Tripathi et al., 2013). Some *Trichoderma* species are characterized by high tolerance to chloroacetanilide herbicides and its effective elimination, such as butachlor (Sherif and Mounir, 2013), or transformation of toxic anilide herbicides byproducts (Cocaign et al., 2013). These benefits make them an important genus of fungi that need to be further investigated for their potential use in the bioremediation of chloroacetanilides.

The aim of this study was to investigate the ability to degrade alachlor and metolachlor by eight filamentous fungi from the genus *Trichoderma*, isolated from different countries (Poland, Russia, Hungary, USA) and located in different microorganism collections. Some of them had been earlier characterized as effective degraders of xenobiotics: pentachlorophenol by *T. citrinoviride* IM 6325 (Szewczyk et al., 2003), alachlor by *T. koningii* IM 0956 (Nykiel-Szymańska et al., 2018), and plastics by *T. virens* DSM 1963 (Klausmeier, 1972). Fungus *T. harzianum* IM 0961 stimulated the growth of wheat seedlings in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) but without 2,4-D removal (Bernat et al., 2018). We also focused on the identification of alachlor and metolachlor degradation metabolites and checked the toxicity of pesticides introduced in fungal liquid cultures. Moreover, the ability of selected *Trichoderma* strains to promote oilseed rape growth under chloroacetanilide herbicide stress was estimated by analyzing siderophore production, phosphate solubilization, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity.

2. Materials and methods

2.1. Chemicals

Alachlor and metolachlor (PESTANAL, analytical standard (99.2%)), ammonium molybdate, ferrous sulfate, α-ketobutyrate, 1-aminocyclopropane-1-carboxylic acid (ACC), 2,4-dinitrophenylhydrazine (DNPH), chrome azurol sulfate (CAS), hexadecyltrimethylammonium bromide (HDTMA) and iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$) were purchased from Sigma-Aldrich (Poznan, Poland). Ethyl acetate was purchased from POCh (Gliwice, Poland), and high-purity solvents used during the sample preparation for high-pressure liquid chromatography (HPLC) were obtained from Sigma-Aldrich (Poznan, Poland) and POCh (Gliwice, Poland).

2.2. Microorganisms and growth conditions

Five fungal strains, including *Trichoderma atroviride* IM QF10, *T. hamatum*

IM I-1, *T. harzianum* IM 0961, *T. koningii* IM 0956, and *T. citrinoviride* IM 6325, were obtained from the Microorganisms Collection of the Department of Industrial Microbiology and Biotechnology, University of Lodz (Lodz, Poland). *T. harzianum* KKP 534 and *T. viride* KKP 792 strains were deposited in the Collection of the Industrial Microorganisms of the Institute of Agricultural and Food Industry (IAFB) (Warsaw, Poland), a member of the World Data Centre for Microorganisms (WDCM 212). One strain of *T. virens* DSM 1963 was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *T. citrinoviride* IM 6325 was isolated from municipal waste (Russia), while the other *Trichoderma* strains were isolated from soil samples (Poland, Hungary, USA).

Seven-day spores of tested *Trichoderma* spp. cultures on ZT agar slants were used to inoculate 20 mL mineral medium with 2% glucose and 1% yeast extracts. The medium was composed of (g L^{-1}): K_2HPO_4 (4.36), KH_2PO_4 (1.7), NH_4Cl (2.1), $MgSO_4 \times 7H_2O$ (0.2), $MnSO_4$ (0.05), $FeSO_4 \times 7H_20$ (0.01), $CaCl_2 \times 2H_2O$ (0.03), glucose (20), yeast extract (10), and distilled water (up to 1 L, pH 6.5).The initial preculture was performed on a rotary shaker (160 rpm) for 24 h at 28 °C in 100 mL Erlenmeyer flasks. The precultures were transferred to a fresh medium in the ratio 1:1 (*v*/v) and incubated for the next 24 h. The homogenous preculture (10%) was introduced into the medium supplemented with alachlor or metolachlor at concentration 50 mg L^{-1} (stock solution 10 mg mL^{-1}). Biotic controls and abiotic controls of the tested herbicides (uninoculated) were also prepared. All cultures were grown for 7 or 14 days on a rotary shaker at 28 °C.

The *Trichoderma* spp. mycelia were separated by filtration with a vacuum pump and then dried at 100 °C to reach a constant weight for dry biomass determination.

2.3. Extraction of chloroacetanilides and LC - MS/MS analysis

The extraction of alachlor or metolachlor from fungal cultures was carried out according to the method described by Nykiel-Szymańska et al. (2018).

Quantitative and qualitative LC-MS/MS analyses of chloroacetanilides were conducted using an Agilent 1200 HPLC system and a QTRAP 3200 mass spectrometer equipped with an ESI source (electrospray ionization). The analysis of the herbicides elimination was performed with the application of the chromatographic method described in our previous study (Nykiel-Szymańska et al., 2018). The detection of the compounds was performed using an MS/MS detector working in the multiple reaction monitoring (MRM) positive ionization mode. The monitored MRM pairs for alachlor were *m/z* 270.1–238.2 and *m/z* 270.1–162.3 at retention time 1.61 min., while the MRM pairs for metolachlor were *m/z* 284.2–252.2 and *m/z* 284.2–176.2 at retention time 1.59 min.

The quantitative analysis of possible alachlor metabolites was conducted with the application of the previously developed method (Nykiel-Szymańska et al., 2018). The measurement of metolachlor metabolites was performed by the IDA method using the precursor ion of *m/z* 176 triggering EPI mass spectra (over a range from *m/z* 50 to 500) in the positive ionization mode.

2.4. Toxicity study

The ecotoxicity of postculture extracts of eight *Trichoderma* strains, cultivated with or without the tested chloroacetanilides for 7 and 14 days, was analyzed by using the acute toxicity test Artoxkit M (MicroBioTests, Inc., Belgium). The bioassay was performed in accordance with the standard operational procedures. The toxicity of the herbicides and their metabolites was calculated as a percentage of *Artemia franciscana* larvae that were not mobile after 24 h of incubation. The motility of larvae was measured microscopically at a magnification of 40×. The larvae that did not move within 10 s were presumed to be dead.

2.5. Influence of Trichoderma strains on rapeseed growth under chloroacetanilides stress

The samples preparation protocol was modified from a procedure developed by Bernat et al. (2018). Rapeseed (*Brassica napus*, cv. Monolit) seeds, obtained from the Regional Center for Sale and Supply of Agriculture, Elewator Sieradz Sp. z o.o., Poland, were germinated on two layers of the paper filter (Whatman No. 1) in Petri dishes (5 cm). Twenty-five seeds were placed on paper filter and, depending on the experimental group, treated with:

(1) distilled water (2.00 mL), (rapeseed without *Trichoderma* spp.)
(2) 1.50 mg L^{-1} alachlor (2.00 mL),
(3) mg L^{-1} metolachlor (2.00 mL),
(4) *Trichoderma* spp. spores (2.00 mL)
(5) *Trichoderma* spp. spores with 1.50 mg L^{-1} alachlor (2.00 mL)
(6) *Trichoderma* spp. spores with 1.00 mg L^{-1} metolachlor (2.00 mL)

A suspension of 1×10^8 *Trichoderma* spp. conidia per mL was prepared. Every 24 h, 1 mL of the diluted (1:4) Hoagland nutrient solution (Hoagland and Arnon, 1950) was added to the Petri dishes to maintain sufficient moisture. Seeds were incubated in a plant growth chamber (IL/750/FIT P Pol-Eko, Poland), under a 16 h light/8 h dark photoperiod, with light supplied by cool white fluorescent lamps (a light intensity of 200 μmol $m^{-2} s^{-1}$). The temperature regime was kept at 22/18 °C for day and night periods, respectively. Relative humidity of the chamber was set to 40%. After 7 days, the shoots and roots were harvested and their length and fresh biomass were estimated.

2.6. Leaf chlorophyll content measurement

The variation of rapeseed leaf chlorophyll content under *Trichoderma* spp. or the herbicide exposure was estimated. The leaf chlorophyll content (mg m^{-2}) was measured using a handheld Chlorophyll Content Meter CCM-300 (Opti Sciences, USA). The measurements were made from one leaf per each seedling ($n = 20$) and the experiments were repeated two separate times. At each evaluation, the content was measured 5 times from the tip to the base and the average was used for analysis.

2.7. Siderophore production by Trichoderma strains

All tested *Trichoderma* strains were screened for siderophore production using the Chrome Azurol sulphonate (CAS) assay described by Schwyn and Neilands (1987). Fungal cultures were grown in iron-deficient medium and incubated for 7 days on a rotary shaker at 28 °C. Quantitative production of siderophore in *Trichoderma* spp. cultures was estimated in 24, 72 and 168 h of incubation. The CAS solution (0.5 mL) was supplemented with 0.5 mL of culture supernatant and incubated for 15 min. To prepare CAS assay solution, 50 mL of 2 mM CAS, 40 mL of 5 mM HDTMA and 10 mL of 1 mM $FeCl_3x6H_2O$ were added. Afterwards, the absorbance of the mixture was measured at 630 nm and calculated by the following formula (Payne, 1993): *siderophore production (%) = (Ar - As)/Ar x 100%,* where Ar represented the absorbance of CAS assay solution and As - the absorbance of a tested sample.

2.8. Evaluation of ACC deaminase in fungal cultures

ACC deaminase activity in liquid cultures of *Trichoderma* spp. was determined according to the method described by Viterbo et al. (2010) with some modifications. Spores suspension of *Trichoderma* spp. (50 μL) was introduced into 10 mL of mineral medium with 2% glucose and incubated for 48 h on a rotary shaker at 28 °C. The accumulated biomass was harvested by centrifugation of the fungal cultures at 8000 *g* for 10 min at 4 °C. Then, the washed biomass was transferred to 5 mL mineral medium with 2% glucose and 3 mM ACC as the sole source of nitrogen and incubated for the next 72 h. After that time *Trichoderma* spp. cultures were homogenized with a ball mill (Retsch MM 400) using 2.5 mL of Tris buffer 0.1 M (pH 8.5). Fifty microliters of toluene was added to 400 μL aliquot of homogenate and vortexed. Twenty microliters of 0.5 M ACC was added to a 200 μL of the toluenized samples incubated for 15 min. at 30 °C. Following the addition of 1 mL of 0.56 M HCl, the mixture was vortexed and centrifuged for 10 min. at 10,000 g at room temperature. One milliliter of the supernatant was mixed with 0.8 mL 0.56 M HCl and 0.3 mL DNPH. After 30 min. of incubation 2 mL of 2 M NaOH was added to the tested samples and then the absorbance was measured at 540 nm. The remaining aliquots of toluenized samples were stored at 4 °C for protein estimation by Bradford (1976) assay. The absorbance of the assay reagents in the presence of ACC was used as reference. The ACCD activity in the tested samples was determined by measuring the amount of α-ketobutyrate generated by the activity of the enzyme. ACC deaminase activity was expressed in μmol of α-ketobutyrate mg^{-1} protein h^{-1}.

2.9. Effect of selected Trichoderma strains on phosphate solubilization

Quantitative estimation of phosphate solubilizing potential in the liquid culture of *Trichoderma* sp. was assayed according to the procedure described by Saravanakumar et al. (2013). Fungal cultures were grown in National Botanical Research Institute Phosphate (NBRIP) broth medium and incubated for 7 days on a rotary shaker at 28 °C. The medium was composed of (g L^{-1}) glucose (10), $Ca_3(PO_4)_2$ (10), $MgCl_2{\cdot}6H_2O$ (5.0), $MgSO_4{\cdot}7H_2O$ (0.25), KCl (0.2) and $(NH_4)_2SO_4$ (0.1) (Nautiyal, 1999). Phosphate solubilization in *Trichoderma* spp. cultures was estimated in 24, 72 and 168 h of incubation. The culture supernatant (0.75 mL) was mixed with 0.75 mL of the reagent containing 1.5% ammonium molybdate, 5.5% sulfuric acid solution and 2.7% ferrous sulfate solution and then the absorbance of the mixture was measured at 600 nm. Phosphate concentration was established by using a standard curve of KH_2PO_4 and expressed in μL mL^{-1}.

2.10. Statistical analysis

All samples were prepared in triplicate and the experiments were repeated two separate times. The samples variability was presented as a standard deviation (± SD). The differences between biomass production by *Trichoderma* spp. under alachlor or metolachlor exposure and the herbicides biotransformation rate were evaluated by two-way ANOVA. The effect of alachlor or metolachlor on root and shoot length, and chlorophyll content of rapeseed seedlings growing alone or in the presence of *Trichoderma* spp. was analyzed using a two-way ANOVA. Siderophore production by tested fungi under alachlor and metolachlor exposure were tested for significant differences by a two-way ANOVA with fixed factors: the effect of the herbicides and incubation time. Data from the toxicity analysis were also analyzed by a two-way ANOVA to achieve the variation attributable to the factors: effect of the herbicides with or without *Trichoderma* spp. and incubation time. Three-way analysis of variance was carried out to evaluate the effect of the tested herbicides, fungal inoculum and incubation time on phosphate solubilization. The means of above-mentioned data were also compared using Tukey's test ($P < .05$).Statistical analyses were performed using the software STATISTICA ver. 13.1 (StatSoft).

3. Results

3.1. Growth of tested Trichoderma strains in the presence of chloroacetanilides

The growth curves of eight *Trichoderma* strains in the presence of alachlor and metolachlor were plotted (Fig. 1A–H). All examined fungi were characterized by high tolerance to the tested concentration of herbicides but chloroacetanilide treatment showed differences in biomass production by the tested *Trichoderma* strains. A two-way ANOVA showed that the effect of the tested chloroacetanilides and incubation time, as well as the interaction between these factors significantly influenced the growth all of the tested fungi ($P < .001$) (Table 1). In the exponential (24 h) phase of growth, alachlor inhibited the growth of *T. koningii* IM 0956, *T. citrinoviride* IM 6325 and *T. viride* KKP 792. Interestingly, a significant growth stimulation in the alachlor supplied fungal stationary cultures was observed in the case of IM 0956 (Fig. 1D), KKP 534 (Fig. 1F), and KKP 792 (Fig. 1G). Biomass production in DSM 1963 cultures supplemented with herbicides was compared with the control growth during the entire period of incubation (Fig. 1H). Metolachlor was the most toxic to *T. harzianum* IM 0961 and inhibited fungal growth during the whole culturing period (Fig. 1B). It also exerted a stronger toxic effect than alachlor on the growth of *Trichoderma* with the exception of IM 0956 and KKP 792 strains, which were more inhibited by alachlor during the initial 24 h of incubation.

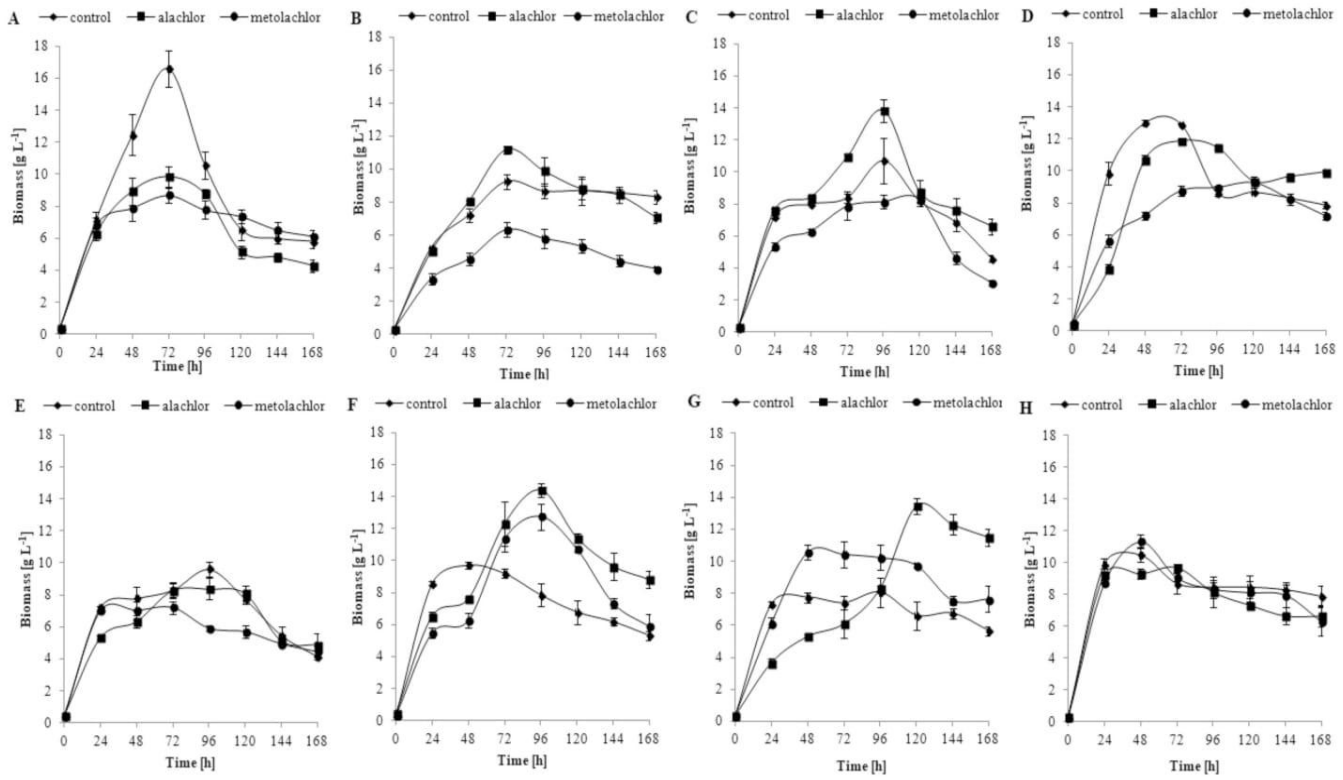

Fig. 1. Growth of the tested *Trichoderma* strains in the presence of chloroacetanilide herbicides in mineral medium supplied with glucose and yeast extracts (A - *T. hamatum* IM I-1; B - *T. harzianum* IM 0961; C - *T. atroviride* IM QF10; D - *T. koningii* IM 0956; E - *T.citrinoviride* IM 6325; F - *T. harzianum* KKP 534;G - *T. viride* KKP 792; H - *T.virens* DSM 1963). Data are the mean ± SD (n = 6).

Table 1
Results from two-way ANOVA for *Trichoderma* spp. growth under chloroacetanilide exposure (H = F value for the effect of chloracetanilides, T = F value for the time of incubation, H × T = F value for interaction between these factors) * indicate significant difference at $P < .001$.

Sample	Alachlor			Metolachlor		
	H	T	H × T	H	T	H × T
IM I-1	443,83*	563,55*	45,92*	309,41*	380,03*	70,86*
IM 0961	12,12*	625,34*	14,90*	1084,50*	419,64*	34,87*
IM QF10	124,83*	510,18*	13,76*	168,67*	427,45*	13,13*
IM 0956	35,7*	3094,9*	442,7*	901,25*	1534,44*	244,95*
IM 6325	26,14*	474,19*	13,84*	169,93*	551,23*	37,84*
KKP 534	388,39*	463,6*	103,35*	61,42*	534,67*	115,01*
KKP 792	185,25*	455,4*	201,37*	219,15*	359,8*	26,24*
DSM 1963	43,42*	469,74*	9,25*	8,63*	442,68*	6,77*

3.2. Degradation and detoxification of chloroacetanilides by Trichoderma strains

The curves of alachlor removal by the studied fungi were plotted using the software Origin Pro 2019 v9.6 (Origin Lab) (Fig. 2A–H). The regression equations, coefficient of determination (R^2) and asymptotic standard errors are presented in Table S1. A two-way ANOVA showed that the fungal inoculum, incubation time, as well as the interaction between these factors significantly influenced alachlor degradation ($P < .001$) (Table 2). All tested microorganisms were characterized by high activity in the elimination of alachlor but the strains differed in the herbicide removal dynamics. As indicated by kinetic data, the most intense decrease in the pesticide content for seven tested strains: IM 6325, KKP 534, IM 0956, IM I-1, IM QF10, IM 0961 and KKP 792 was observed during the initial 72 h of incubation. The above-mentioned strains eliminated 50–85% and 80–99% of alachlor after 24 and 72 h of incubation, respectively. Among the tested fungal strains, *T. virens* DSM 1963 exhibited the lowest degradation potential (Fig. 2H). In this case, efficient alachlor removal started after 24 h and finally led to an 80% reduction in the herbicide amount. Based on the mass weight and fragmentation patterns of alachlor metabolites, eight by-products were identified: *N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide (**1**); (Z)-1-[(2,6-diethylphenyl)-*N*-(methoxymethyl)amino]ethene-1,2-diol (**2**); *N*-(2,6-diethylphenyl)-*N*-[(dihydroxymethoxy)methyl]-2-methoxyacetamide (**3**); *N*-(2,6-diethylphenyl)-2-hydroxy-*N*-(methoxymethyl)acetamide (**4**); 2,6-diethyl-*N*-methylaniline (**5**); *N*-(2,6-diethylphenyl)-2,2-dihydroxy-*N*-[(methoxy)methyl]acetamide (**6**); *N*-(2,6-diethylphenyl)-*N*-[(dihydroxymethoxy)methyl]-2-hydroxy-2-methoxyacetamide (**7**) and *N*-(2,6-diethylphenyl)-2,2-dihydroxy-*N*-[(hydroxymethoxy)methyl]acetamide (**8**). The chemical structures of alachlor metabolites and their time-dependent conversion are presented in Fig. S1A and Table S2, respectively.

The tested filamentous fungi were also capable of effectively utilizing another chloroacetanilide herbicide - metolachlor. The curves of metolachlor decompostion by tested *Trichoderma* spp. were plotted using the software Origin Pro 2019 v9.6 (Origin Lab) (Fig. 2A–H). The regression equations, coefficient of determination (R^2) and asymptotic standard errors are presented in Table S1. A two-way ANOVA indicated that the fungal inoculum, incubation time, as well as the interaction between these factors significantly influenced metolachlor degradation ($P < .001$) (Table 2). The biodegradation kinetics of metolachlor also revealed that there were differences in the herbicide decomposition potential among the tested fungi. Moreover, the comparison chloroacetanilides removal rateindicated that metolachlor was slower degraded than alachlor by all fungal strains. The most substantial depletion in the pesticide content was noted for *T. harzianum* IM 0961, *T. virens* DSM 1963, *T. atroviride* IM QF 10 and *T. viride* KKP 792 between 72 h and 120 h of incubation. The results indicated that metolachlor degradation by *T. harzianum* IM 0961, *T. virens* DSM 1963, *T. citrinoviride* IM 6325, *T. hamatum* IM I-1 and *T. atroviride* QF 10 was more effective in comparison to other strains. After 7 days the above-mentioned *Trichoderma* strains eliminated approximately 71–79% of the herbicide, whereas *T. viride* KKP 792, *T. harzianum* KKP 534, and *T. koningii* IM 0956 were only able to reduce 40–62% of the initial concentration. The intermediate 2-chloro-N-(2-ethyl-6-methylphenyl)-N-

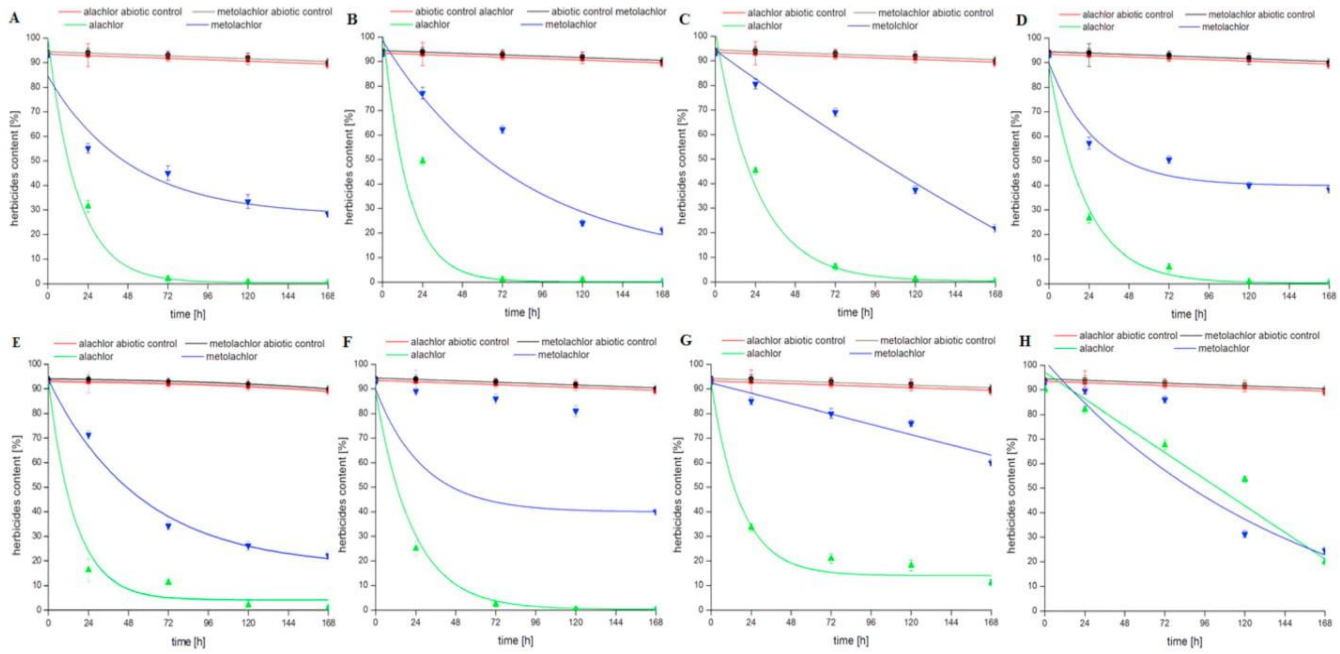

Fig. 2. Alachlor and metolachlor removal by tested *Trichoderma* strains in mineral medium extracts supplied with glucose and yeast extracts (■/● – alachlor/metolachlor abiotic control, respectively, ▲ – alachlor, ▼ - metolachlor) (A - *T. hamatum* IM I-1; B - *T. harzianum* IM 0961; C - *T. atroviride* IM QF10; D - *T. koningii* IM 0956; E - *T.citrinoviride* IM 6325; F - *T. harzianum* KKP 534;G - *T. viride* KKP 792; H - *T.virens* DSM 1963). Data are the mean ± SD (n = 6).

Table 2
Results from two-way ANOVA for chloroacetanilides removal by *Trichoderma* spp. (F = F value for the fungal inoculum, T = F value for the time of incubation, F × T = F value for interaction between these factors). * indicate significant difference at $P < .001$.

Sample	Alachlor			Metolachlor		
	F	T	F × T	F	T	F × T
IM I-1	1704605*	130789*	11768*	224546*	19629*	4068,81*
IM 0961	1260167*	116325*	35609*	159749*	27288*	12695*
IM QF10	1265868*	110820*	38634*	76318*	15228*	17875*
IM 0956	845325*	62322*	37013*	262984*	22197*	10075*
IM 6325	1244127*	87098*	35855*	666573*	78806*	22782*
KKP 534	891296*	65126*	33917*	7840*	4208*	3632*
KKP 792	75530*	1164829*	26459*	6098*	1353*	1560*
DSM 1963	152717*	33617*	9502*	62782*	21948*	20578*

(2-hydroxy-1-methylethyl)acetamide (**2**) was the main product of metolachlor biodegradation identified in fungal cultures during 168 h. When the cultivation period was extended, N-(2-ethyl-6-methylphenyl)-2-hydroxy-N-(2-hydroxy-1methylethyl)acetamide (**1**), N-(2-ethyl-6-methylphenyl)-2-hydroxy-N-(2-hydroxy-1-methylethyl)acetamide (**3**) were also detected. In the case of *T. harzianum* KKP 534 another known metabolite N-(2-ethyl-6-methylphenyl)-2-hydroxy-(2-methoxy-1-methylethyl)acetamide (**4**) dominated in metolachlor transformation. The chemical structures of metolachlor metabolites and their time-dependent conversion are presented in Fig. S1B and Table S3 in Supplementary Data. Moreover, the summary of qualitative analysis is shown in Table S4.

An Artoxkit M bioassay was performed to test whether detoxification occurred during chloroacetanilides degradation by the tested *Trichoderma* strains (Table 3). After the incubation for 7 and 14 days, the extracts of the abiotic controls containing 50 mg L^{-1} alachlor or 50 mg L^{-1} metolachlor inhibited the motility of *A. franciscana* larvae by 90.75 ± 0.98, 88 ± 1.41, 88.50 ± 9.00, and 83.33% ± 10.00, respectively. The motility inhibition of the tested crustaceans by pure alachlor and metolachlor solution (without incubation and extraction) in both cases reached 100%. The toxicity of biotic controls originating from *T. hamatum* IM I-1, *T. koningii* IM 0956, *T. harzianum* IM 0961 and KKP 534 cultures (without the herbicide), observed in the tested organism, was negligible (1–8%). A two-way ANOVA showed significant differences between the effect of the herbicides with or without *Trichoderma* spp. ($F = 123{,}386$, $P < .001$ in the case of alachlor and $F = 15{,}215$, $P < .001$ in the case of metolachlor), incubation time ($F = 1782$, $P < .001$ and $F = 28{,}770$, $P < .001$, respectively), and interaction between these factors ($F = 921$, $P < .001$ and $F = 3054$, $P < .001$, respectively). In the case of extracts from the biotic controls and alachlor-treated cultures of *T. atroviride* IM QF10 and *T. citrinoviride* IM 6325, a high (90–95%) motility inhibition of *A. franciscana* was observed. Prolongation of the above-mentioned fungal cultures up to 14 days did not reduce herbicide toxicity. Biotransformation of alachlor by *T. viride* KKP 792, *T. harzianum* KKP 534, and *T. virens* DSM 1963 led to 53–80% reduction in toxicity. The immobilization of *A. franciscana* after 24 h of incubation, with extracts from alachlor-treated *T. koningii* IM 0956, *T. harzianum* IM 0961, and *T. hamatum* IM I-1 and samples, reached 6.67 ± 1.04, 4.17% ± 0.28, and 3.71 ± 0.18 (for a 7-day degradation study) and 5.56 ± 1.33, 3.71% ± 0.28, and 3.33 ± 0.92 (within 14 days), respectively. The results of Artoxkit M tests indicated a decrease in metolachlor toxicity due to biotransformation by IM I-1, IM 0956, IM 0961, KKP 534, and KKP 792 strains. A comparison of the motility inhibition for the abiotic control and xenobiotic-treated fungal samples indicated an almost 1.5–2.5-fold reduction in toxicity for 14-day cultures after 24 h of incubation. High toxicity (70–80%) was observed for 7-and 14-day extracts from IM 6325, IM QF10, and DSM 1963 cultures supplemented with metolachlor.

3.3. Rapeseed seedlings growth in the presence of Trichoderma spp. and herbicides

In this study, we also investigated the effect of alachlor at concentration 1.5 mg L^{-1} and metolachlor at 1.0 mg L^{-1} on the growth of the seedlings inoculated with *Trichoderma* strains. Higher concentrations of alachlor and metolachlor induced a strong inhibition of seed germination (data not shown). The used concentration of chloroacetanilides reduced the growth of rapeseed roots and shoots, although not significantly ($P = .42$, $P = .97$ for rapeseed roots and $P = .99$, $P = .95$ for shoots treated with alachlor and metolachlor, respectively). The effect of the tested *Trichoderma* spp. on rapeseed

Table 3
Toxicity analysis of eight *Trichoderma* spp. cultures with alachlor or metolachlor in the Artoxkit M assay. Data are the mean ± SD ($n = 6$). Different letters indicate significant difference ($P < .001$) according to two-way ANOVA followed by Tukey's HSD post-hoc test.

Sample	Immobilization of *A. franciscana* [%]					
	Biotic control		Alachlor		Metolachlor	
	7 days	14 days	7 days	14 days	7 days	14 days
Abiotic control	–	–	88.9 ± 1.41^{a}	90.75 ± 0.98^{a}	88.50 ± 9.00^{a}	83.33 ± 10.00^{a}
Pure herbicide solution	–	–	100 ± 0.2	100 ± 0.2	100 ± 0.50	100 ± 0.50
IM I-1	8.33 ± 0.94^{b}	3.33 ± 0.58^{b}	3.71 ± 0.18^{b}	3.33 ± 0.92^{b}	66.10 ± 9.20^{ac}	56.05 ± 6.94^{cd}
IM QF 10	99.00 ± 1.41^{a}	92.00 ± 5.66^{a}	91.67 ± 5.42^{a}	93.27 ± 3.53^{a}	81.00 ± 5.00^{a}	78.00 ± 5.60^{b}
IM 0956	3.03 ± 0.77^{b}	2.78 ± 0.23^{b}	6.67 ± 1.33^{b}	5.56 ± 1.04 ^{b}	65.30 ± 2.42^{aed}	46.70 ± 5.80^{c}
IM 0961	5.80 ± 0.28^{b}	4.17 ± 1.36^{b}	4.17 ± 0.90^{b}	3.71 ± 0.28^{b}	51.51 ± 3.20^{ce}	34.44 ± 0.96^{f}
IM 6325	96.67 ± 4.20^{a}	85.00 ± 4.14^{a}	96.29 ± 1.14^{a}	96.67 ± 2.10^{a}	88.89 ± 9.25^{a}	78.40 ± 7.30^{a}
KKP 534	7.50 ± 1.44^{b}	1.00 ± 0.03^{b}	20.74 ± 1.08^{g}	20.00 ± 1.00^{g}	71.74 ± 8.79^{ah}	59.33 ± 1.15^{d}
KKP 792	38.5 ± 7.83^{d}	14.44 ± 2.48^{bg}	47.69 ± 2.07^{ce}	34.81 ± 5.00^{cf}	100 ± 0.02^{a}	46.67 ± 5.77^{ci}
DSM 1963	20.00 ± 0.20^{g}	12.73 ± 0.69^{g}	74.30 ± 3.95^{a}	54.85 ± 5.00^{cehi}	81.48 ± 6.20^{a}	67.70 ± 5.00^{ai}

seedling growth, fresh biomass weight, and leaf chlorophyll content in seedlings untreated and treated with alachlor and metolachlor was estimated after 7 days. The interaction between the effect of the herbicides and fungal inoculum significantly influenced roots and shoots length of rapeseed seedlings ($F = 5.48$, $P < .001$ for roots and $F = 5.91$, $P < .001$ for shoots), which was demonstrated in the two-way ANOVA test. The results indicated that *T. harzianum* KKP 534 and *T. viride* KKP 792 increased the length of the roots by 38 and 45%, respectively. At the same time, the other strains had no significant effect on the roots of seedlings untreated with herbicides, but under chloroacetanilides pressure the inoculation with *T. koningii* IM 0956, *T. harzianum* KKP 534 and *T. virens* DSM 1963 spores resulted in the elongation of roots with statistical importance ($P < .001$) (Table 4). On the other hand, the presence of *T. koningii* IM 0956, *T. harzianum* KKP 534, *T. viride* KKP 792, and *T. virens* DSM 1963 significantly increased the length of shoots (Table 4). In addition, an increase in the fresh biomass of rapeseed seedlings was observed after the treatment with *T. koningii* IM 0956 for control (4.02 g ± 0.10) and alachlor supplied plants (3.87 g ± 0.21) (Table S5). The results revealed that *T. koningii* IM 0956 had the biggest influence on the growth and weight of fresh biomass in seedlings treated with alachlor and metolachlor. The shoot length of the plants treated with fungi and alachlor or metolachlor increased by 80 and 50%, respectively, while the root length increased by 37 and 36%, respectively, and the fresh weight rose by 82 and 31%, respectively. The results presented in Table 4 show that the chlorophyll content in seedling leaves treated with chloroacetanilides was lower, although without statistical significance. The two-way ANOVA results demonstrated that the interaction between the effect of the herbicides and fungal inoculum significantly affected chlorophyll content in rapeseed seedlings ($F = 4.16$, $P < .001$). Application of *T. hamatum* IM I-1 had a positive effect on the chlorophyll content (94.50 mg m^{-2} ± 9.27) in comparison to control seedlings untreated with herbicides (76.10 mg m^{-2} ± 7.75) A statistically significant increase in the chlorophyll content was also observed for rapeseed seedlings exposed to metolachlor protected by all *Trichoderma strains*, except of *T. viride* IM 792 and exposed to alachlor inoculated with *T. harzianum* IM 0961 (84.07 mg m^{-2} ± 9.66) and *T. koningii* IM 0956 (82.20 ± 6.35 mg m^{-2}) spores in comparison to seedlings exposed to the herbicide without *Trichoderma* spp.(68.10 mg m^{-2} ± 8.12).

3.4. Evaluation of plant growth-promotion (PGP) characteristics of *Trichoderma spp.*

To evaluate the potential of the tested *Trichoderma* spp. in promoting plant growth under herbicide-stressed conditions, siderophore production, phosphorus solubilization, and ACC deaminase activity in fungal cultures were examined. Siderophore production by chloroacetanilide-tolerant *Trichoderma* strains in fungal liquid cultures with the herbicides was estimated (Fig. 3). The interaction between the incubation time and the exposure to the herbicides significantly influenced siderophore production by *Trichoderma* spp. ($F = 1570$, $P < .001$), which was demonstrated in the two-way ANOVA test. The formation of siderophores in control and fungal cultures supplied with the herbicides occurred during the initial 24 h and reached the maximum at 72 h of incubation. Supplementation with alachlor or metolachlor resulted in the inhibition of siderophore production in the cultures of *T. koningii* IM 0956, *T. citrinoviride* IM 6325, *T. harzianum* IM 0961,*T. hamatum* IM I-1 and *T. harzianum* KKP 534 during the first 24 h of incubation. After this period, the formation of siderophores in the herbicide-treated cultures of *T. hamatum* IM I-1, *T. koningii* IM 0956, *T. citrinoviride* IM 6325 and *T. atroviride* IM QF10 strains was compared with the control fungal cultures. Moreover, in the stationary (72 h) phase of growth a stimulation of the production of iron-chelating compounds was observed in the cultures of *T. harzianum* IM 0961 supplemented with alachlor and metolachlor and also in alachlor-treated cultures of *T. hamatum* IM I-1, *T. viride* KKP 792, and *T. virens* DSM 1963.

As shown in Fig. 4, a significant increase in soluble phosphate concentration in all the fungal strains compared to abiotic control was observed from 24 to 168 h of incubation ($P < .001$). A three-way ANOVA indicated that the interaction between the incubation time, exposure to the herbicides, and fungal inoculum significantly influenced phosphate solubilization ($F = 74$, $P < .001$). The concentration of soluble phosphate varied from 132 to 164 mg L^{-1} in the fungal culture filtrates in comparison to 13–18 mg L^{-1} in the filtrates from the abiotic control. The P-solubilizing capacity of *Trichoderma* strains in the presence of alachlor and metolachlor was also assayed (Fig. 4). The inhibiting effect of herbicides in *T. harzianum* KKP 534, *T. virens* DSM 1963, and *T. viride* KKP 792 cultures was observed during the entire incubation period. Phosphate solubilization by these strains was the most significantly decreased in 24 h alachlor and metolachlor supplied cultures, by 85, 65,50% and 86, 58, 53%, respectively ($P < .001$).

Some *Trichoderma* spp. were found to have the ability to produce ACC deaminase, an enzyme involved in ethylene synthesis (Viterbo et al., 2010). Four tested *Trichoderma* strains with the highest capability to effectively eliminate tested chloroacetanilides and PGP activities in the presence of the herbicides, were also examined for ACCD activity (Table 5). The ACC deaminase average activity of selected *Trichoderma* strains IM 0961, IM 0956, KKP 534 and IM 6325 cultures, with ACC as the sole nitrogen source, was found to be 52.14 ± 1.35, 16.10 ± 0.10, 12.55 ± 2.18 and 8.21 ± 0.29 µmol α-ketobutyrate mg^{-1} protein h^{-1}, respectively, whereas in alachlor- and metolachlor-treated cultures of IM 0961, the activity of the enzyme significantly increased by 66 and 55%, respectively ($P < .001$) (86.64 ± 0.13 in alachlor-

Table 4
Effect of alachlor (1.5 mg L^{-1}) and metolachlor (1 mg L^{-1}) treatment on the average root and shoot length, and chlorophyll content of rapeseed seedlings growing alone or in the presence of *Trichoderma* strains. Data are the mean ± SD (n = 20). Different letters indicate significant difference ($P < .001$) according to two-way ANOVA followed by Tukey's HSD post-hoc test.

Sample	Root length [mm]			Shoot length [mm]			Chlorophyll content [mg m^{-2}]		
	Control	Alachlor	Metolachlor	Control	Alachlor	Metolachlor	Control	Alachlor	Metolachlor
Rapeseed without *Trichoderma* spp.	56.55 ± 4.35^{a,b,c}	55.17 ± 4.93^{b,c}	51.90 ± 6.47^{c,i}	14.40 ± 1.52^{a,b}	12.11 ± 1.15^{b}	12.75 ± 1.39^{b}	76.10 ± 7.75^{a,b,c}	68.10 ± 8.12^{b,c}	61.53 ± 8.59^{c}
IM QF10	66.50 ± 6.42^{a,e}	54.10 ± 5.17^{b,c,f}	56.50 ± 4.76^{a,b,c,f}	17.00 ± 1.73^{a,d,f}	13.40 ± 2.66^{b}	12.65 ± 0.94^{b}	73.00 ± 8.59^{a,b,c}	64.12 ± 7.91^{a,b,c}	74.33 ± 5.70^{a,b,c}
IM I-1	66.05 ± 6.49^{a,f,g}	53.50 ± 6.55^{b,c,f}	52.10 ± 5.08^{b,c,f}	14.80 ± 1.22^{a,b}	14.20 ± 1.54^{a,b}	13.00 ± 2.21^{b}	94.50 ± 9.27^{d}	72.09 ± 9.65^{a,b,c}	84.08 ± 10.82^{a,d}
IM 0956	65.60 ± 3.60^{a,f,g,h}	75.70 ± 7.21^{d,e,h}	70.60 ± 8.35^{e,g,f}	18.10 ± 1.72^{c,f}	21.90 ± 3.97^{d,e}	19.10 ± 1.20^{c,d}	88.00 ± 7.97^{a,d}	82.20 ± 6.35^{a,d}	84.50 ± 7.48^{a,d}
IM 0961	61.75 ± 7.21^{a,g}	59.45 ± 3.27^{b,c}	54.50 ± 7.81^{a,b,c}	14.91 ± 1.76^{a,b}	13.65 ± 1.44^{b}	14.20 ± 1.41^{a,b}	77.60 ± 8.66^{a,b}	84.07 ± 9.66^{a,d}	81.50 ± 9.59^{a,b}
IM 6325	60.75 ± 2.26^{a,b,c,g}	58.3 ± 5.70^{a,b,c}	60.35 ± 6.33^{a,b,c}	13.75 ± 1.70^{b,g}	16.10 ± 1.59^{a,f,g}	13.05 ± 1.33^{b}	82.89 ± 7.43^{a,d}	80.83 ± 7.77^{a,b}	80.55 ± 8.67^{a,b}
KKP 534	81.80 ± 3.86^{d}	75.90 ± 5.30^{d,e}	76.80 ± 3.83^{d,e}	22.30 ± 2.26^{c,e}	17.80 ± 1.71^{c,d}	18.62 ± 2.01^{c,d}	73.44 ± 9.33^{a,b,c}	76.00 ± 8.54^{a,b,c}	77.89 ± 5.64^{a,b}
KKP 792	78.50 ± 8.00^{d,e}	69.60 ± 7.39^{d,e,g}	56.60 ± 4.92^{a,b,c}	19.30 ± 1.70^{c,d}	18.00 ± 2.13^{c,d}	20.10 ± 1.91^{c,d}	75.93 ± 6.47^{a,b,c}	80.93 ± 8.30^{a,b}	67.00 ± 4.71^{a,b,c}
DSM 1963	60.10 ± 2.34^{a,b,g}	69.50 ± 3.50^{d,e,g}	64.00 ± 1.97^{a}	19.50 ± 0.99^{c,d}	19.70 ± 0.94^{c,d}	17.70 ± 1.34^{c,d}	76.67 ± 8.79^{a,b}	75.44 ± 8.16^{a,b,c}	71.00 ± 8.78^{a,b,c}

treated samples and 80.83 ± 1.89 µmol α-ketobutyrate mg^{-1} protein h^{-1} in metolachlor-treated samples). At the same time, the ACCD activity in the IM 0956 culture with the addition of herbicides was compared with that of control (17.30 ± 1.62, 14.45 ± 2.12 µmol α-ketobutyrate mg^{-1} protein h^{-1} in alachlor- and metolachlor-treated cultures, respectively).

4. Discussion

Trichoderma strains are known for their ability to eliminate different hazardous pollutants, including heavy metals, pesticides or PAHs (Tripathi et al., 2013). Our former study indicated that *T. koningii* IM 0956 was capable of efficiently removing alachlor (Nykiel-Szymańska et al., 2018), which prompted us to examine the process of alachlor and metolachlor decomposition by strains from the genus *Trichoderma* originating from different microbial collections. Some of these strains were also characterized by the ability to degrade various xenobiotics such as plastics and PCP (Klausmeier, 1972; Szewczyk et al., 2003). In the present study, the growth of the tested *Trichoderma* strains varied with the addition of chloroacetanilide herbicides. Alachlor stimulated the biomass production in the stationary cultures of IM QF10, IM 0956, KKP 534, and KKP 792. According to literature data, microorganisms able to degrade toxic compounds may use them as a source of biogenic elements for their physiological processes (Kodama et al., 2001; Moneke, 2010; Bera and Ghosh, 2013). Growth inhibition under the same concentration of alachlor was reported for *Paecilomyces marquandii* (Słaba et al., 2013) and *Candida xestobii* (Munoz et al., 2011). Metolachlor exhibited the largest effect on IM 0961 growth during the entire period of incubation. The study conducted by Sahid and Kasim (1994) and Ismail and Shamsuddin (2005) demonstrated a higher inhibitory effect of metolachlor on the growth of soil filamentous fungi compared with alachlor, whereas in the case of *C. xestobii*, only a slight reduction in biomass growth was observed under 50 mg L^{-1} metolachlor exposure (Munoz et al., 2011). In this study, it was not possible to state clearly which of the tested herbicides was more toxic in relation to the examined fungal model. The sensitivity to chloroacetanilides was an individual characteristic of *Trichoderma* strains. However, literature data indicated that in the case of higher organisms (rat, mouse, rabbit, fish), alachlor is more toxic than metolachlor (US EPA, 1995, 1998). All the examined filamentous fungi were capable of effective elimination of alachlor or metolachlor but showed different rates in the decomposition of herbicides. The results indicated that alachlor biodegradation rate was very fast for five *Trichoderma* strains IM QF10, IM 6325, IM I-1, IM 0961, and IM 0956 from the culture collection of our Department and after the initial 72 h of cultivation, the herbicide removal ranged from 80 to 90%. After 168 h of cultivation, only a negligible amount of alachlor was detected. The lowest capacity of xenobiotic decomposition by the tested strains was reported for DSM 1963 and KKP 792 strains (80 and 89% after 168 h, respectively). Comparison of the results presented in most papers exploring microbial transformation of alachlor with the data for *Trichoderma* spp. tested in our study showed a faster rate of herbicide decomposition by *Trichoderma*. Previous reports showed that *P. marquandii* (Słaba et al., 2013) and *C. xestobii* (Munoz et al., 2011) were able to eliminate only 70% of 50 mg L^{-1} alachlor after 3 days of incubation, respectively. Six *Streptomyces* strains degraded about 60–75% of the herbicide at an initial concentration of 144 mg L^{-1} after 14 days. On the other hand, the rate of metolachlor biotransformation by the tested *Trichoderma* strains was slower than that of alachlor and the most efficient removal occurred between 72 and 120 h of incubation. After 7 days of cultivation, IM 0956, IM 0961, IM QF10, IM I-1, IM 6325, KKP 534, and DSM 1963 strains were able to degrade 62–79% of the herbicide, while the KKP 792 strain eliminated only 40% of metolachlor. A similar rate of the herbicide decomposition at initial concentration 50 mg L^{-1} has been recorded for *Fusarium* spp., *Mucor racemosus* (Saxena et al., 1987) and *C. xestobii* (Munoz et al., 2011). Sanyal and Kulshrestha (2003) reported that *Aspergillus flavus*

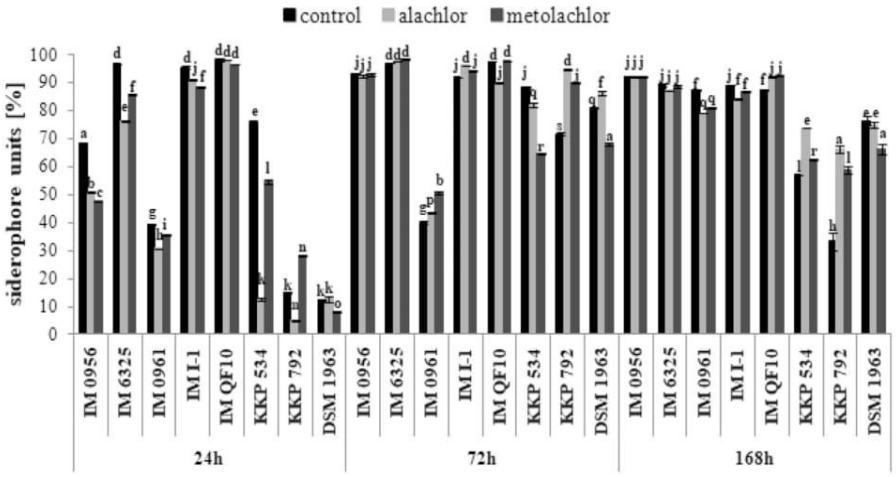

Fig. 3. Production of siderophore by the tested *Trichoderma* spp. Data are the mean ± SD ($n = 6$). Different letters indicate significant difference ($P < .001$) according to two-way ANOVA followed by Tukey's HSD post-hoc test.

and *A. terricola* degraded 68.76% and 44.12% of metolachlor at an initial concentration of 20 mg L^{-1} after 7 days of growth, respectively. According to Zhang et al. (2011) and Hou et al. (2014), the molecular structure of chloroacetanilides affects their biodegradability. Compounds with an alkoxymethyl side chain on amide nitrogen (e.g., alachlor, acetochlor, butachlor) are more rapidly degraded than compounds with an alkoxyethyl side chain on amide nitrogen (e.g., metolachlor, pretilachlor), which may explain the lower rate of metolachlor decomposition by the tested strains compared with that of alachlor. Alachlor biotransformation by *Trichoderma* spp. starts with oxidative dechlorination and then the metabolites undergo hydroxylation of terminal carbon atoms of hydroxyacetaldehyde substituents and dimethyl ether, which creates various di- or tri-hydroxylated by-products. Alachlor transformation via N-alkyl group hydroxylation was described for *P. marquandii* (Słaba et al., 2013), whereas in the earlier work, only hydroxylation of ethyl chains was detected (Pothuluri et al., 1993). This work proved that both ways of substrate hydroxylation are common in fungal cells. The predominant metabolism of metolachlor by the tested *Trichoderma* strains included *O*-demethylation of the N-alkyl substituent to form 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-hydroxy-1-methylethyl)acetamide (**2**). This mechanism has already been described in the literature for *Chaetomium globosum* (McGahen and Tiedje, 1978), *Cunninghamella elegans* (Pothuluri et al., 1997), *Fusarium* spp., *Mucor racemosus*, or bacteria such as *Bacillus circulans* and *B. megaterium* (Krause et al., 1985). Similarly, the production of metabolites 1 and 3 began with *O*-demethylation and next two-way dechlorination occurred. Oxidative dechlorination resulted in the formation of N-(2-ethyl-6-methylphenyl)-2-hydroxy-N-(2-hydroxy-1methylethyl)acetamide (**1**), and reductive dechlorination resulted in the formation of N-(2-ethyl-6-methylphenyl)-N-(2-hydroxy-1-methylethyl)acetamide (**3**). Microbial conversion of metolachlor via the above-mentioned types of reactions has not been described so far.

Table 5
ACCD activity in *Trichoderma* spp. cultures with or without chloroacetanilide herbicides (A—alachlor, M—metolachlor). * indicates values that differ significantly from the biotic control (IM 0961) at $P < .001$.

Treatment	ACCD activity [μM α-ketobutyrate mg^{-1} protein h^{-1}]
IM 0961	52.14 ± 1.35
IM 0956	16.10 ± 0.10
KKP 534	12.55 ± 2.18
IM 6325	8.21 ± 0.29
IM 0961 + A	86.64 ± 0.13*
IM 0961 + M	80.83 ± 1.89*
IM 0956 + A	17.30 ± 1.62
IM 0956 + M	14.45 ± 2.12

Toxicity estimation of metabolites formed during xenobiotics biotransformation is a major concern of ecotoxicology, because generated by-products may exhibit either lower or higher toxicity than the parent compounds (Bernat et al., 2013; Souissi et al., 2013). However, microbiological degradation of toxic compounds involving hydroxylation and dechlorination reactions can lead to the detoxification of the substrate (Pothuluri et al., 1993, 1997). The Artoxkit M bioassay used in this study showed a nearly 100% reduction in toxicity in comparison to the rate of alachlor biodegradation by *T. harzianum* IM 0961, *T. koningii* IM 0956, and *T. hamatum* IM I-1. In the case of KKP 534, KKP 792, and DSM 1963 incubated with alachlor, the immobilization of *A. franciscana*

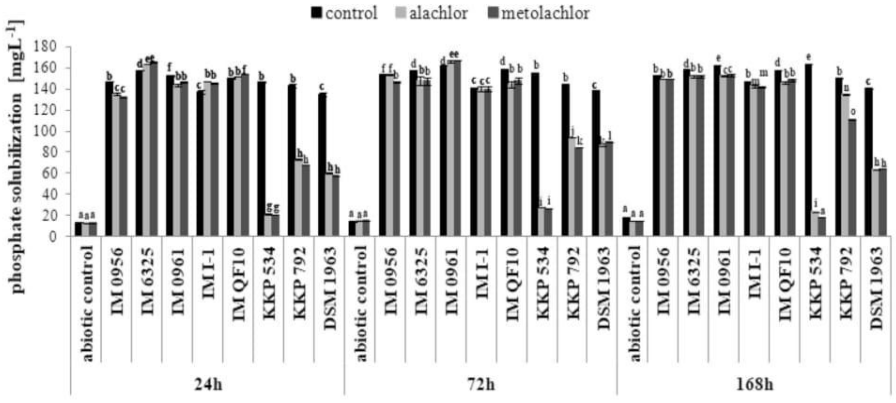

Fig. 4. Phosphate solubilization by the tested *Trichoderma* spp. Data are the mean ± SD ($n = 6$). Different letters indicate significant difference ($P < .001$) according to three-way ANOVA followed by Tukey's HSD post-hoc test.

was in the range of 20–50%, whereas alachlor extract or pure solution of herbicide at the same concentration caused 88.9 and 100% immobilization of crustacean, respectively. The obtained results clearly indicate the detoxification of the herbicide by these strains. Immobilization of the tested invertebrates in metolachlor-treated samples of IM I-1, IM 0956, IM 0961, KKP 534, and KKP 792 showed a 1.5–2.5 fold reduction in toxicity compared with the parent compound. To the best of our knowledge, this is the first report that demonstrates the detoxification of alachlor and metolachlor during the microbial degradation by different *Trichoderma* strains.

The high mortality of *A. franciscana* observed in the samples originating from the fungal cultures IM QF10 and IM 6325 untreated with the herbicide may be associated with the generation of secondary by-products showing antimicrobial activity and the also have deleterious effect on aquatic invertebrates *A. fransciscana* and *D. magna* (Favilla et al., 2006; Thiruchchelvan et al., 2013). Moreover, in the postculture extracts of IM QF10 and IM 6325 were identified secondary metabolites with antimicrobial activity like harzianic acid, harziaphilic acid, T22azaphilone, T39butenolide and trichoharzin (data not shown). Biocontrol activity of two *Trichoderma* strains can enhance growth of plants exposed to phytopathogens.

The extensive application of herbicides may adversely affect both the growing plants protected by them and soil microorganisms (Ahemad and Kibret, 2014). It has been reported that some *Trichoderma* sp. are known for their beneficial effect on plant growth and resistance to abiotic and biotic stress factors (Viterbo et al., 2010; Zhang et al., 2016). In the present study, the *Trichoderma* strains KKP 534 and KKP 792 had the ability to stimulate growth of seedling roots and shoots, while elongation of shoots was observed in the case of IM 0956 and DSM 1963. It has been suggested that the production of phytohormone - indole-3-acetic acids is related to the promotion of plant growth by some *Trichoderma* spp. (Hermosa et al., 2012). The improvement in seedling growth by supplementation of *Trichoderma* was noticed especially in the presence of alachlor or metolachlor. The growth promotion in seedlings was more related with fungal PGP activities than alachlor or metolachlor decomposition by the tested strains, because in the samples containing rapeseed seedlings inoculated with *Trichoderma* spores, herbicide elimination did not exceed 30% (data not shown). In addition, plant inoculation with IM 0956 conidia significantly enhanced the fresh weight of seedlings. Bernat et al. (2018) reported that the application of *T. harzianum* IM 0961 increased the growth of wheat seedlings only in the presence of 2.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid. However, the inoculation of *T. atroviride* IM QF10 had no beneficial effect on the growth of rapeseed seedlings, whereas a positive effect of *T. citrinoviride* IM 6325 appeared only in the presence of the herbicides. This confirmed that the interaction between fungi and plants to stimulate plant growth is a species-dependent ability of *Trichoderma* spp. (Nieto-Jacobo et al., 2017). Determination of plant growth promoting characteristics of tested *Trichoderma* strains revealed presence of phosphate solubilization, siderophore production and ACC deaminase activity. Our study demonstrated that all eight *Trichoderma* strains were able to solubilize insoluble tricalcium phosphate (TCP) to various extents. From 24 to 168 h of incubation, the concentration of solubilized phosphate gradually increased in the culture filtrates from the biotic control of the tested strains. A similar content of P-solubilized was observed in the cultures of ten *Trichoderma* strains isolated from the rhizosphere of *Avicennia marina* (Saravanakumar et al., 2013). A study conducted by Rudresh et al. (2005) also confirmed the ability of another *Trichoderma* strain to dissolve TCP but *T. viride* PDBCTV 32, *T. virens* PDBCTVs 12, and *T. virens* PDBCTVs 13 were found to solubilize about 5% of the phosphate dissolved by the strains used in this study. The amount of P-solubilized decreased in fungal cultures supplemented with chloroacetanilides. Alachlor and metolachlor showed the most deleterious effect on phosphate solubilization in KKP 534, KKP 792, and DSM 1963 cultures in comparison to the cultures not supplemented with the herbicides. A decline in phosphate solubilizing potential of a large variety of microorganisms under xenobiotic exposure had previously been mentioned (Ahemad and Khan, 2010, 2011a,b). Moreover, all the tested *Trichoderma* strains exhibited the ability to produce siderophores both in the absence and presence of chloroacetanilides. The addition of the herbicides to the medium reduced significantly the amount of siderophores formed by the cultures of almost all the tested strains at 24 h of growth. However, after this period, the effect of the herbicides was marginally inhibitory and in the case of KKP 792 and IM 0961, the production of siderophores was significantly induced. This unexpected result could have been caused by interactions of fungal metabolism with metal trace ions other than iron nd with the herbicides. Manganese and iron compounds may impact the fate of organic pollutants. These processes depend on solution chemistry and microbial activity (Borch et al., 2010). On the other hand, metals other than iron, in the proper form and concentration, may also enhance siderophores production (Saha et al., 2012). Similarly to the harmful effect of alachlor and metolachlor on P-solubilization, the production of siderophores by IM 6325, KKP 534, and KKP 792 decreased after 24 h of incubation. The reduction in the of the amount iron-chelating molecules in the cultures of plant growth-promoting bacteria under various pesticide exposure had been described earlier (Ahemad and Khan, 2011a,b, 2012). The inhibition of siderophore production and P-solubilizing potential in the cultures of KKP 534, KKP 792, and DSM 1963 supplemented with alachlor or metolachlor can be related to the damage to or decreased functioning of protein or pathways responsible for these PGP traits.

The ACC deaminase activity was found in all the selected *Trichoderma* spp. but the highest activity was observed in *T. harzianum* IM 0961, about 3–6 fold in comparison to the other strains. However, in the case of IM 0956, IM 6325, and KKP 534, the ACC deaminase activity was compared with that reported for *T. asperellum* T206, *T. atroviride*, *T. harzianum* TSK8, and *T. estonicum* SKS1 (8.20–13.50 µmol α-ketobutyrate mg^{-1} protein h^{-1}) (Gravel et al., 2007; Saravanakumar et al., 2018; Viterbo et al., 2010). Moreover, our results revealed that the addition of alachlor or metolachlor to fungal cultures led to an increase in the enzyme activity in *T. harzianum* IM 0961. Similarly, a higher ACCD activity in plant growth-promoting bacteria was observed under heavy metal (Pb, As, Cu, Cd or Zn) exposure (Carlos et al., 2016; Grobelak et al., 2018). According to Glick (2014), ACC deaminase is present in PGP microorganisms at a relatively low level but its activity may be stimulated under biotic or abiotic stress (high salinity, heavy metal, pesticide) and thus can be helpful in minimizing stress.

5. Conclusion

The present study demonstrated the ability of eight *Trichoderma* strains to transform and detoxify popular chloroacetanilide herbicides such as alachlor and metolachlor. The rapid and effective removal of the tested herbicides by *Trichoderma* spp. can be used for future strategies of bioremediation of agricultural areas polluted with residues of chloroacetanilides. This is particularly important especially in the case of alachlor, which is banned in the EU due to its endocrine-disrupting activity. The pathway of chloroacetanilides degradation by *Trichoderma* species involves mainly dechlorination and hydroxylation reactions. N-(2-ethyl-6-methylphenyl)-2-hydroxy-N-(2-hydroxy-1-methylethyl)acetamide and N-(2-ethyl-6-methylphenyl)-N-(2-hydroxy-1-methylethyl) acetamide were identified for the first time as metolachlor metabolites formed by fungi. Our study showed that selected *Trichoderma* strains enhanced the growth of rapeseed seedlings under alachlor or metolachlor exposure and mitigated the harmful effect of these herbicides on plants by stimulating various PGP traits such as siderophore production, phosphate solubilization, and ACC deaminase activity. Among the tested *Trichoderma* strains, *T. koningii* IM 0956 is the most interesting research model because of the high ability to eliminate tested chloroacetanilides and multiple PGP activities even in the presence of the herbicides. In the future, it would be necessary to check whether the

strains of *Trichoderma*, capable of degrading herbicides in the laboratory scale, will not mitigate the effectiveness of chloroacetanilides action in crop protection in agricultural soils.

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Compliance with ethical standards

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2019.11.018.

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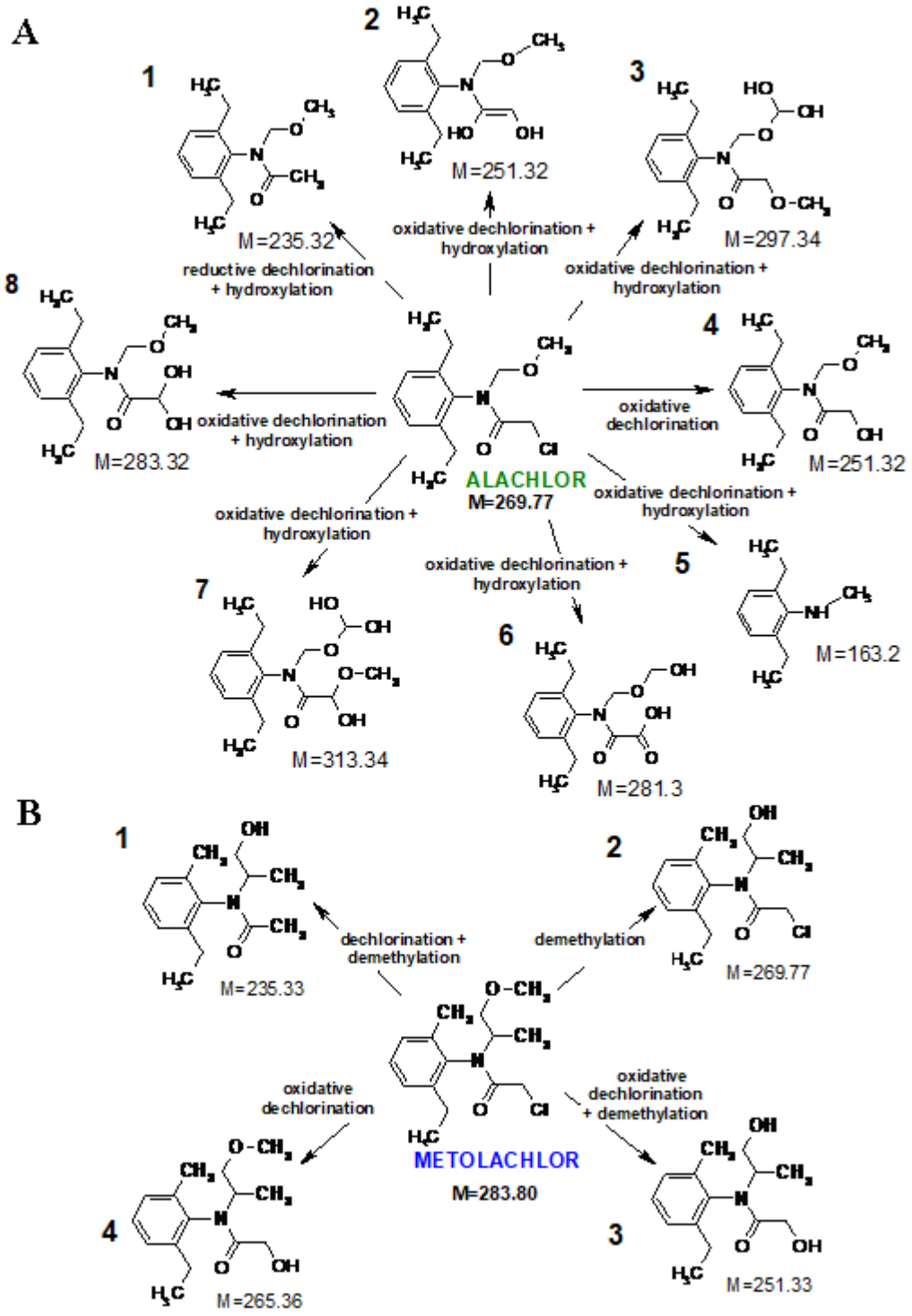

Fig. S1 The chemical structure of alachlor (**A**) and metolachlor (**B**) by-products formed by the tested *Trichoderma* strains.

Table S1 Alachlor metabolites and their time dependent conversion (*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide (**1**); (Z)-1-[(2,6-diethylphenyl)-*N*-(methoxymethyl)amino]ethene-1,2-diol (**2**); *N*-(2,6-diethylphenyl)-*N*-[(dihydroxymethoxy)methyl]-2-methoxyacetamide (**3**); *N*-(2,6-diethylphenyl)-2-hydroxy-*N*-(methoxymethyl)acetamide (**4**); 2,6-diethyl-*N*-methylaniline (**5**); *N*-(2,6-diethylphenyl)-2,2-dihydroxy-*N*-[(methoxy)methyl]acetamide (**6**); *N*-(2,6-diethylphenyl)-*N*-[(dihydroxymethoxy)methyl]-2-hydroxy-2-methoxyacetamide (**7**) and *N*-(2,6-diethylphenyl)-2,2-dihydroxy-*N*-[(hydroxymethoxy)methyl]acetamide (**8**)).

Sample	**Time [h]**		
	72	**120**	**168**
IM QF10	1; 3; 5	1; 3; 4; 5; 6	1; 3; 4; 5; 6
IM I-1	1; 5	1; 4; 5	1; 4; 5
IM 0956	1; 3; 5; 6	1; 2; 5; 6; 7	1; 2; 3; 4; 5; 6; 7
IM 0961	1; 4; 5	1; 2; 4; 5	1; 2; 4; 5
IM 6325	1; 5; 6	1; 2; 4; 5; 6	1; 2; 3; 4; 5; 6; 7
KKP 534	1; 3; 5; 8	1; 4; 5; 6; 8	4; 5; 6; 8
KKP 792	1; 3; 5; 8	1; 3; 4; 5; 7; 8	1; 5; 3
DSM 1963	1; 4; 5	1; 4; 5; 7	1; 4; 5; 7

Table S2 Metolachlor metabolites and their time dependent conversion (N-(2-ethyl-6-methylphenyl)-N-(2-hydroxy-1-methylethyl)acetamide (**1**), 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-hydroxy-1-methylethyl)acetamide (**2**), N-(2-ethyl-6-methylphenyl)-2-hydroxy-N-(2-hydroxy-1-methylethyl)acetamide (**3**), and N-(2-ethyl-6-methylphenyl)-2-hydroxy-(2-methoxy-1-methylethyl)acetamide (**4**)) .

Sample	**Time [h]**		
	168	**240**	**336**
IM QF10	2	1; 2	1; 2
IM I-1	2	1; 2	1; 2
IM 0956	1; 2	1; 2; 3	1; 2; 3
IM 0961	2	1; 2	1; 2
IM 6325	2	1; 2	1; 2
KKP 534	4	1; 3; 4	1; 3; 4
KKP 792	2	1; 2; 3	1; 2; 3
DSM 1963	2	1; 2	1; 2

Table S3. Mass spectra, molecular weights and retention times of 4 detected metolachlor metabolites produced by tested fungi.

N-(2-ethyl-6-methylphenyl)-N-(2-hydroxy-1-methylethyl)acetamide			2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-hydroxy-1-methylethyl)acetamide			N-(2-ethyl-6-methylphenyl)-2-hydroxy-N-(2-hydroxy-1-methylethyl)acetamide			N-(2-ethyl-6-methylphenyl)-2-hydroxy-(2-methoxy-1-methylethyl)acetamide		
No.	1		**No.**	2		**No.**	3		**No.**	4	
RT (min)	1.22		**RT (min)**	1.64		**RT (min)**	1.71		**RT (min)**	1.80	
Formula	$C_{14}H_{21}NO_2$		**Formula**	$C_{12}H_{20}ClNO_2$		**Formula**	$C_{15}H_{21}NO_3$		**Formula**	$C_{14}H_{21}NO_3$	
MW	235.33		**MW**	269.77		**MW**	265.36		**MW**	251.33	
Mass spectrum			**Mass spectrum**			**Mass spectrum**			**Mass spectrum**		
m/z	**rel.int. (%)**		**m/z**	**rel.int. (%)**		**m/z**	**rel.int. (%)**		**m/z**	**rel.int. (%)**	
121.1	22.63		109.62	3.1		134.82	6.81		91.14	1.29	
134.04	5.19		121.10	4.96		136.26	77.27		104.16	2.58	
146.2	7.93		134.16	4.96		146.16	6.81		119.04	2.15	
148.26	33.02		149.20	9.31		148.20	36.36		132.00	1.29	
149.20	22.63		161.88	4.34		176.34	100		134.11	15.51	
161.20	14.82		176.28	100		204.18	81.81		135.42	1.29	
176.20	100		185.10	54		205.50	20.45		146.4	3.01	
207.30	11.31		195.12	17.39		233.40	79.54		148.08	3.87	
234.30	68.80		213.20	78.88		266.26	6.81		161.04	1.72	
235.30	100		214.10	14.28					176.22	75.43	
236.90	8.25		252.18	7.45					184.26	2.58	
			253.20	94.4					252.30	39.65	
			271.14	13.66							

Table S4. Effect of alachlor (1.5 mg L^{-1}) and metolachlor (1 mg L^{-1}) treatment on the dry weight of rapeseed seedlings growing alone or in the presence of *Trichoderma* strains. Data were evaluated with a one-way ANOVA followed by Tukey's post-hoc test, each value is the mean ± SD (*n* = 20). **a** indicates values that differ significantly from the control treatment (without *Trichoderma* spp.) at $P < 0.001$. **b** indicates values that differ significantly from the treatment with alachlor (without *Trichoderma* spp).

Sample	Total fresh weight [g]		
	Control	Alachlor	Metolachlor
Rapeseed without *Trichoderma* sp.	2.36±0.18	2.10±0.19	2.12±0.18
IM QF10	3.48±0.44	2.26±0.36	2.56±0.35
IM I-1	3.17±0.76	2.21±0.06	2.53±0.26
IM 0956	4.02±0.10[a]	3.87±0.21[b]	2.79±0.85
IM 0961	3.01±0.10	2.45±0.20	2.44±0.10
IM 6325	2.57±0.15	2.25±0.24	2.27±0.20
KKP 534	2.39±0.02	2.03±0.76	2.70±0.12
KKP 792	2.78±0.36	1.67±0.37	1.93±0.66
DSM 1963	2.81±0.34	1.10±0.15	1.55±0.05

Artykuł naukowy nr 2

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Potential of *Trichoderma koningii* to eliminate alachlor in the presence of copper ions

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ABSTRACT

The filamentous fungus *Trichoderma koningii* is capable of fast and effective eliminate alachlor (90% after 72 h when added separately and 80–60% in the presence of 1–5 mM of copper). After 168 h over 99% elimination of alachlor resulted in detoxification and was connected with the mitigation of reactive oxygen species (ROS) production. Using MS/MS techniques, seven dechlorinated and hydroxylated metabolites were identified. Cytochrome P450 and laccase participate in biotransformation of the herbicide by this non-ligninolytic fungus. Laccase activity is stimulated both by copper and the mixture of copper and alachlor, which seems to be important for combined pollutants. *T. koningii* is characterized by high tolerance to copper (up to 7.5 mM). The metal content in mycelia reached 0.9–7.76 mg in 1 g of dry biomass. Our results suggest that *T. koningii* strain seems to be a promising tool for bioremediation of agricultural areas co-contaminated with copper-based fungicides and chloroacetanilide herbicides.

1. Introduction

Alachlor [2-chloro-*N*-2,6-diethylphenyl-*N*-(methomethyl)acetamide] is a chloroacetanilide herbicide registered for pre- and post-emergent control of annual grasses and broadleaf weeds of corn, soybeans, cotton, sorghum, peanuts, rapeseed, or beans. Its huge consumption has caused negative impacts on both agricultural ecosystems and aquatic environment (Rattanawong et al., 2015). Alachlor toxicity is primarily due to its good solubility in water (240 mg L^{-1}), which contributes to its migration in the environment. Penetration of this compound into surface water and groundwater poses a threat to living organisms. Alachlor has been classified by Environmental Protection Agency (EPA, US) as a potential carcinogen of B2 group (EPA 1998). Furthermore, due to its toxic and genotoxic properties, the herbicide has been included in the group of compounds disrupting proper functioning of the endocrine system of humans and animals (EDC's) (UNEP and WHO, 2012). Therefore, there is a great concern regarding the fate of this herbicide and its byproducts in the environment. Alachlor can be degraded through physicochemical methods, but microbial degradation has a crucial relevance in its transformation in the natural environment. In the polluted areas, degradation is performed by a selected group of microflora that are capable of surviving in the presence of toxic substances (Wang et al., 2008; Xu et al., 2008). Till date, various microorganisms that are capable of degrading alachlor under both aerobic and anaerobic conditions have been isolated and identified. A lot of attention has been paid to bacteria originating from acetanilide-contaminated sludge and soil. Most of them belong to *Agrobacterium* sp., *Ancylobacter* sp., *Burkholderia* sp. (Ewida, 2014), *Bacillus* sp. (Wang et al., 2008; Xu et al., 2008), *Microbacterium* sp., *Pseudomonas* sp. (Xu et al., 2008) and *Streptomyces* sp. (Sette et al., 2004). However, only a limited number of studies have involved selected filamentous fungi that can effectively remove alachlor, for example, *Phanerochaete chrysosporium* (Ferrey et al., 1994; Chirnside et al., 2011), *Cunninghamella elegans* (Pothuluri et al., 1993), *Chaetomium globosum* (Tiedje and Hagedorn, 1975), *Paecilomyces marquandii* (Słaba et al., 2013c), *Candida xestobii* (Munoz et al., 2011), and *Coriolus versicolor* (Hai et al., 2012).

Xenobiotics degradation by soil microflora can be limited by the presence of excessive concentrations of heavy metal ions. Copper is one of the heavy metals having an adverse effect on the microbial processes such as cellular polymer synthesis, respiration, and cell division. In addition, it disturbs enzyme activity, membrane permeability, and can induce oxidative stress (Iwinski et al., 2017; Słaba et al., 2013a, 2013b). Copper compounds are often used as effective fungicides to protect infected plants as they are highly effective against fungi (Filimonova et al., 2018). Since 1904, copper has also been used as an aquatic herbicide and algaecide (Iwinski et al., 2017). In contrast, it is a beneficial micronutrient needed to achieve the appropriate height and

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quality in the case of many crops (Zou et al., 2015).

Nonspecific oxidative enzymes, for example, laccase, lignin peroxidase, manganese peroxidase, and cytochrome P450 oxidase are often engaged in the fungal elimination of many xenobiotics e.g., polycyclic aromatic hydrocarbons (PAHs) (Bautista et al., 2015), chlorophenols, pesticides (Rao et al., 2014) and dyes (Jasińska et al., 2015). Laccase activity in non-ligninolytic fungi from the genus *Trichoderma* has been described by Chakroun et al. (2010); Sadhasivam et al. (2008), and Wang et al. (2012). Fungal laccases have been used in many processes, for example, decolorization or synthesis of dye (Jasińska et al., 2015; Polak and Jarosz-Wilkolazka, 2012), biopulping, delignification of ligninocellulosis compounds, production of ethanol, treatment of wastewater (Chen et al., 2015b), and degradation of pesticides (Khambhaty et al., 2015). Elimination of alachlor with the participation of the enzyme laccase was observed in the lignin-degrading white rot fungus *P. chrysosporium* (Chirnside et al., 2011), whereas degradation of chloroacetanilides by non-ligninolitic fungi with laccase is so far undocumented. The role of cytochrome P450 (CYP450) in alachlor metabolism was detected in different eukaryotic organisms such as fungi (*C. elegans*) (Pothuluri et al., 1993) and mammalians (rats) (Kale et al., 2008).

Filamentous fungi from the genus *Trichoderma* are cosmopolitan microorganisms, mostly isolated from the soil or plant roots These fungi are best known for supporting the growth of plants and protecting them from pathogens (Tripathi et al., 2013). Their prevalence in the environment is primarily due to their ability to grow rapidly, to propagate in adverse environmental conditions, as well as their ability to use different sources of carbon and nitrogen (Raspanti et al., 2009). Because of their high metabolic activity, the fungi are able to degrade a broad spectrum of xenobiotics, for example PAHs, cyanides, synthetic dyes, cresols, phenols, and plant protection products (e.g., dicofol, dichlorodiphenyltrichloroethane (DDT), dichlorvos, and chlorpyrifos) (Tripathi et al., 2013).

In this study, we investigated the transformation and detoxification of alachlor by *Trichoderma koningii* with the involvement of laccase and the cytochrome P450 system. Moreover, due to the high tolerance of *T. koningii* to copper ions, its ability to simultaneously eliminate alachlor and copper ions was determined.

2. Materials and methods

2.1. Chemicals

Alachlor (PESTANAL®, analytical standard (99.2%)), metabolic inhibitors, laccase substrate – 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), copper sulfate, nitrotetrazolium blue chloride (NBT) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Sigma-Aldrich (Poznan, Poland). Ethyl acetate, for alachlor extraction, was purchased from POCh (Gliwice, Poland), and high purity solvents used during sample preparation for high pressure liquid chromatography (HPLC) were obtained from Sigma-Aldrich (Poznan, Poland) and POCh (Gliwice, Poland).

2.2. Microorganism and growth conditions

The filamentous fungus *Trichoderma koningii* IM 0956 was obtained from the Department of Industrial Microbiology and Biotechnology, Institute of Microbiology, Biotechnology and Immunology, University of Lodz, Poland. Seven-day-old spores of *T. koningii* strain from cultures on ZT agar plants (g L^{-1}: glucose (4); Difco yeast extract (4); agar (25); and malt extract (6 °Blg), up to 1 L; pH 7.0) were used to inoculate 20 mL of mineral medium with 2% glucose and 1% yeast extract in 100-mL Erlenmeyer flasks. The medium was composed of (g L^{-1}): K_2HPO_4 (4.36), KH_2PO_4 (1.7), NH_4Cl (2.1), $MgSO_4 \times 7H_2O$ (0.2), $MnSO_4$ (0.05), $FeSO_4 \times 7H_20$ (0.01), $CaCl_2 \times 2H_2O$ (0.03), glucose (20), yeast extract (10), and distilled water (up to 1 L, pH 6.5). The fungal cultures were grown on a rotary shaker (160 rpm) for 24 h at 28 °C. The preculture was transferred to a fresh medium in the ratio of 1:1 and incubated for the next 24 h. The homogenous preculture (10%) was introduced into the medium supplemented with 50 mg L^{-1} alachlor dissolved in ethanol (stock solution 10 mg mL^{-1}) or a control culture without the tested compound. In addition, abiotic controls (uninoculated) were also prepared. All cultures were incubated at 28 °C on a rotary shaker (160 rpm). At appropriate time, culture samples were selected for analyses.

2.3. Determination of alachlor and its metabolites

Samples were prepared according to the method described by Słaba et al. (2013c) with some modifications. The cultures were homogenized with glass beads twice for 4 min at 25 m s^{-1} (Retsch, Ball Mill MM 400). Next, the samples were extracted twice with ethyl acetate. The extracts were dried with anhydrous sodium sulfate and evaporated under reduced pressure at 40 °C. Evaporated residues were dissolved in 2 mL of methanol and 0.2 mL was transferred to chromatography plates for quantitative and qualitative analyses.

Quantitative analysis of alachlor was performed on the Agilent 1200 LC System coupled with an AB Sciex 3200 QTRAP mass detector equipped with a TurboSpray Ion Source (electrospray ionization (ESI)). The separation was performed in a capillary Eclipse XDB-C18 column (50 mm × 4.6 mm, 4.6 μm) maintained at 37 °C. The mobile phase consisted of water (A) and methanol (B) supplemented with 5 mmol L^{-1} ammonium formate. The run time was 6 min with the solvent gradient initiated at 50% B. After 1 min, during the next minute, B was decreased to 5% and maintained at 5% for four additional minutes before returning to the initial solvent composition over 2 min. The flow rate was 600 μL min^{-1}. The detection of alachlor was performed on an MS/MS detector working in the multiple reaction monitoring (MRM) positive ionization mode. The monitored MRM pairs were *m/z* 270.1–238.2 and *m/z* 270.1–162.3 at 1.61 retention time. The quantitative analysis of alachlor in the tested samples was performed according to the standard equation ($y = 6.67e+0.006\times$; $r = 0.991$), and the alachlor content was calculated in percentage. A 100% value represented the amounts of alachlor, which were estimated in abiotic controls.

Samples for qualitative analysis were selected from 0, 24, 72, 120, and 168 h of culture and included corresponding biotic and abiotic controls acting as a reference for the detection of alachlor metabolites. The same chromatographic method was used to identify the possible alachlor metabolites, which were detected in previously extracted samples. The mass spectrometric analysis was performed using a targeted scanning mode. According the previous data on alachlor biodegradation (Słaba et al., 2013c), a list of predicted Multiple Reaction Monitoring (pMRM) pairs of possible metabolites in positive ionization mode was created. The pMRM-based method was constructed based on the characteristic fragmentations occurring in the alachlor mass spectrum: *m/z* 238, *m/z* 224, *m/z* 220, *m/z* 210, *m/z* 208, *m/z* 90, and *m/z* 77. Then, Information Dependent Acquisition (IDA) criteria were employed to automatically trigger the acquisition of full-scan MS/MS spectra for any compounds that were detected by the pMRM scans. The Enhanced Product Ion (EPI) scan-type was used to acquire full-scan MS/MS spectra in the range of *m/z* 50–350 in the linear ion trap of the QTRAP system. The optimized ion source parameters were: CUR: 25, IS: 5500 V, TEMP: 550 °C, GS1: 55 psi, GS2: 50 psi.

2.4. Toxicity study

The possible toxicity of postculture extracts of the *T. koningii* strain, cultivated with or without alachlor was examined using a commercial bioassay Artoxkit M provided by MicroBioTests, Inc., Belgium. Experiments were based on the immobilization of the larvae of *Artemia franciscana* in accordance with standard operational procedures. The

fungal cultures (with and without the herbicide after 7 and 14 days) were homogenized using a ball mill. The homogenized samples and abiotic controls were extracted twice with ethyl acetate, dried with anhydrous sulfate, and evaporated under reduced pressure at 40 °C. The extracts were dissolved in 0.1 mL of ethanol and diluted with saline water to obtain 20 mL of the initial volume of the cultures. Next, appropriate dilutions were prepared. *A. franciscana* controls with saline water and with the same volume of ethanol as in the test samples were also prepared. The toxicity of alachlor and its metabolites was calculated as the percentage of immobile larvae after 24 h of incubation. The motility of larvae was measured microscopically at a magnification of 40×. The larvae that did not move within 10 s were presumed to be dead.

2.5. Superoxide anion and nitrogen oxide detection

The detection of superoxide anion ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$) in *T. koningii* hyphae was carried out according to the detailed method described in the paper by Siewiera et al. (2017).

2.6. Laccase activity and effect of copper on enzyme production

Laccase activity in the centrifuged supernatant was assayed, by monitoring ABTS oxidation at 420 nm, according to the procedure described by Jasińska et al. (2015).

To establish if the presence of copper and alachlor in the medium would affect the activity of laccase, the liquid culture of *T. koningii* was supplemented with various concentrations of $CuSO_4$ (1, 2.5, 5, 7.5, and 10 mM) and 50 mg L^{-1} alachlor. The samples were collected every 24 h for 7 days and residual activities were determined using the ABTS assay.

2.7. Cytochrome P450 and laccase inhibition studies

CYP450 inhibiting compounds (proadifen, SKF 525-A (0.1 mM), and 1-aminobenzotriazole, ABT (0.25 mM)) or a supposed laccase inhibitor (sodium azide, NaN_3 (0.1 mM)) were introduced to 18 mL growth medium supplemented with alachlor (50 mg L^{-1}) and inoculated with *T. koningii*. Then, the flasks were incubated in the same conditions on a rotary shaker (160 rpm) at 28 °C. At appropriate time, culture samples were selected for analyses and prepared according to the method described in Section 2.3.

2.8. Alachlor elimination in the presence of copper

Liquid cultures of *T. koningii* with the addition of 50 mg L^{-1} alachlor and different concentrations of copper (1.0, 2.5, and 5.0 mM) were incubated on a rotary shaker (160 rpm) at 28 °C. At appropriate time, culture samples were selected for analyses and prepared according to the method described in Section 2.3.

2.9. Metal content in fungal mycelium

Copper content in the mycelium, after thermal mineralization by nitric acid (65%), was determined in an atomic absorption spectrometer Spectra 240 FS (Agilent). The quantity of metal loaded in the mycelium was expressed as milligrams of metal per gram of dry weight of mycelium.

2.10. Statistical analysis

Results were assessed by ANOVA and statistical analyses were performed on three replicates of data obtained from each treatment. The significance ($P < 0.05$) of differences was treated statistically by one- or two-way ANOVA followed by Tukey's test.

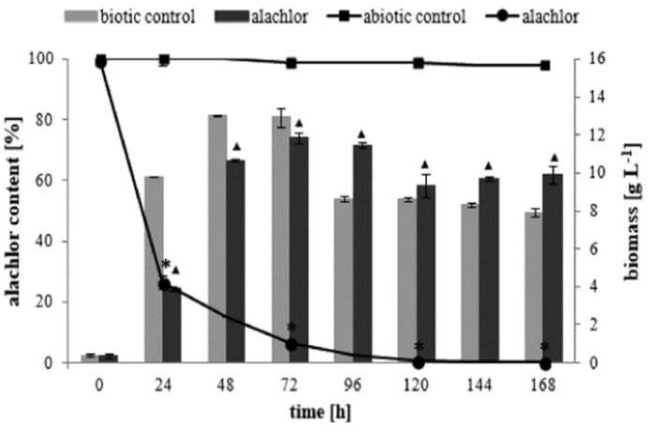

Fig. 1. *Trichoderma koningii* growth (grey column - biotic control, black column - alachlor) and alachlor elimination (line with black squares - abiotic control, line with black circles - alachlor) in rich mineral medium supplied with glucose and yeast extract. Data are presented as mean ± SD (n = 3). * Indicates values that differ significantly from the abiotic control at $P < 0.001$. ▲ Indicates values that differ significantly from the biotic control at $P < 0.001$.

3. Results and discussion

3.1. Growth, alachlor degradation, and detoxification in fungal liquid cultures

Our preliminary results indicate a high activity of selected *Trichoderma* strains in the elimination of alachlor (data not shown). Among *Trichoderma* strains isolated from environments contaminated with inorganic and organic pollutants, only *T. koningii* revealed a capacity to degrade alachlor with substrate-induced laccase activity (Table S1). The influence of alachlor at the concentration of 50 mg L^{-1} on the growth of the *T. koningii* strain in rich liquid mineral medium with glucose (2%) and yeast extract (1%) has been illustrated in Fig. 1. The tested concentration of alachlor inhibited significant the growth of the fungus during 72 h of incubation (strongly within first 24 h) ($P < 0.05$). After this time the growth stimulation by the herbicide in *T. koningii* stationary cultures was observed. On the other hand the same concentration of the herbicide resulted in the toxic effect on fungal growth throughout the culture period in the case of *P. marquandii* and *C. xestobii* (Munoz et al., 2011; Słaba et al., 2013c).

The dynamics of alachlor elimination by *T. koningii* is also shown in Fig. 1. No such rapid alachlor degradation rate in fungal cultures had been previously recorded as for *T. koningii* examined in this study. After initial 24-h incubation, 72% of the supplemented alachlor was removed, and after 72 h, more than 90%. In the case of the filamentous fungus *P. marquandii* and the yeast *C. xestobii*, 30% of alachlor was detected after 3 days of incubation (Munoz et al., 2011; Słaba et al., 2013c). A two-way ANOVA results showed that the fungal inoculum and time as well as their interaction significantly influenced on alachlor degradation ($F = 391.7$, $P < 0.001$; $F = 73748$, $P < 0.001$ and $F = 316.2$, $P < 0.001$, respectively). Based on the alachlor metabolites mass weight and fragmentation patterns, seven byproducts were identified: *N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide; (*Z*)-1-[(2,6-diethylphenyl)-*N*-(methoxymethyl)amino]ethene-1,2-diol; *N*-(2,6-diethylphenyl)-*N*-[(dihydroxymethoxy)methyl]-2-methoxyacetamide; *N*-(2,6-diethylphenyl)-2-hydroxy-*N*-(methoxymethyl)acetamide; *N*-(2,6-diethylphenyl)-2,2-dihydroxy-*N*-[(methoxy)methyl]acetamide; 2,6-diethyl-*N*-methylaniline and *N*-(2,6-diethylphenyl)-*N*-[(dihydroxymethoxy)methyl]-2-hydroxy-2-methoxyacetamide. Examples of mass spectra and structural formulas of alachlor intermediates are presented in Fig. 2. *T. koningii* transformed alachlor mainly via oxidative dechlorination and *N*-acetyl hydroxylation, but reductive dechlorination resulting in the formation of *N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide was also noted. Alachlor by

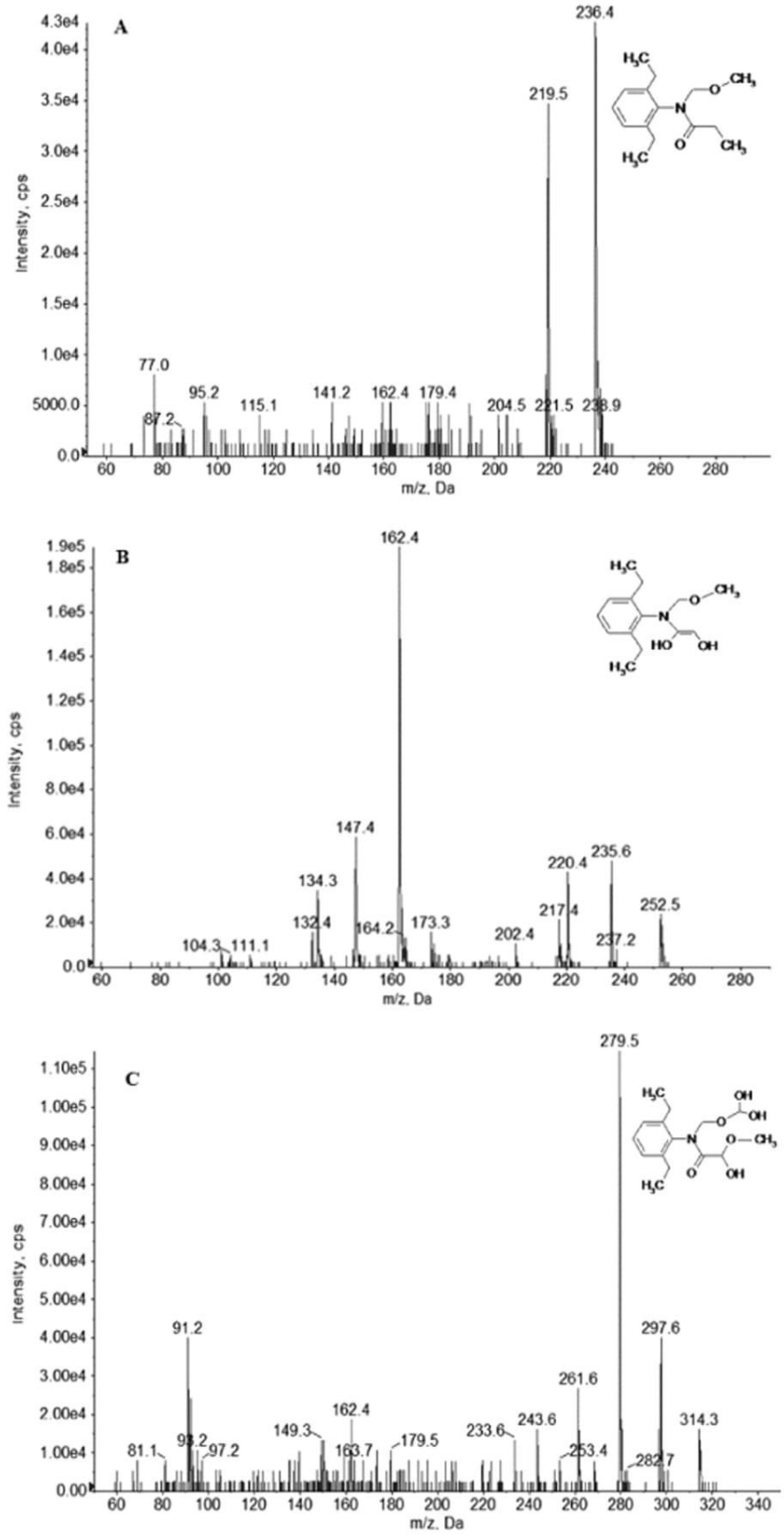

Fig. 2. Mass spectra and chemical structures of exemplary alachlor byproducts originating from *Trichoderma koningii* cultures detected by LC-MS/MS. (**A**) N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide; (**B**) (*Z*)-1-[(2,6-diethylphenyl)-*N*-(methoxymethyl)amino]ethene-1,2-diol; (**C**) *N*-(2,6-diethylphenyl)-*N*-[(dihydroxymethoxy)methyl]-2-hydroxy-2-methoxyacetamide.

products detected in *Trichoderma* had been earlier described for another ascomycete fungus, *P. marquandii* (Słaba et al., 2013c). The obtained results revealed that the mechanism of alachlor transformation in microscopic fungi via *N*-alkyl group hydroxylation leading to the formation of mono-, di-, or trihydroxylated byproducts is more popular than it was supposed. Earlier, preferential hydroxylation of the arylethyl chain of alachlor was observed in *Cunninghamella elegans* (Pothuluri et al., 1993). On the other hand, only in a few works, the

Table 1
Toxicity of extracts obtained from *Trichoderma koningii* cultures supplemented with alachlor against *Artemia franciscana*.

Type of postculture extracts	Immobilization of *A. franciscana* [%]	
	7 days	14 days
Abiotic control	90.75 ± 0.98	88.00 ± 1.41
T. koningii (biotic control)	3.03 ± 0.77	2.78 ± 0.23
T. koningii + alachlor	6.67 ± 1.04*	5.56 ± 1.33*
Pure alachlor solution (without incubation and extraction)	100 ± 0.20	100 ± 0.20

* Indicates values that differ significantly from the abiotic control at $P < 0.001$, each value is the mean ± SD (n = 3).

modern MS/MS methods were used to identify alachlor metabolites in microbial cells and to propose degradation pathway (Sette et al., 2004; Słaba et al., 2013c).

Biodegradation is the most efficient way of eliminating xenobiotics from soil, but it can also lead to the formation and accumulation of toxic and carcinogenic intermediate metabolites (Bernat et al., 2013). An Artoxkit M bioassay was performed to test if the detoxification occurred during alachlor degradation by *T. koningii*. Extracts of the abiotic controls incubated for 7 and 14 days, containing alachlor at a concentration of 50 mg L^{-1} inhibited the motility of *A. franciscana* larvae in 90.75 ± 0.98% and 88 ± 1.41%, respectively (Table 1). The inhibition of motility by pure alachlor solution (without incubation and extraction) reached 100 ± 0.2%. Toxicity of biotic controls originating from *Trichoderma* cultures (without the herbicide), observed in the tested organism, was negligible. Two-way ANOVA showed significant differences among the effect of alachlor treated samples with or without *T. koningii* ($F = 618.3$, $P < 0.001$). However, there was no significant effect of incubation time ($F = 0.13$, $P = 0.72$) (7 and 14 days) and no interaction between incubation time and alachlor treated samples with or without *T. koningii* ($F = 0.46$, $P = 0.64$). The immobilization of *A. franciscana* after 24 h of incubation with extracts obtained from alachlor-treated fungal samples, reached 6.67 ± 1.04% (for a 7-day degradation study) and 5.56 ± 1.33% (within 14 days). The obtained results showed a 13-fold reduction in toxicity. This is the first report showing almost total detoxification of alachlor via fungal transformation. Only a slight reduction in alachlor solution toxicity during direct ozonation and O_3/H_2O_2 advanced oxidation was obtained (Qiang et al., 2010). Interestingly, although alachlor oxidation by *P. marquandii*, proceeded probably along the same route, there was no decrease in toxicity against *A. franciscana*. The 24-h EC_{50} of alachlor amounted to 8.17 ± 1.11 and 7.35 ± 2.54 mg L^{-1} for abiotic and alachlor-treated fungal samples, respectively (Słaba et al., 2013c). Probably, a crucial role in the final toxicity of alachlor supplied fungal cultures can play concentrations of particular byproducts.

3.2. ROS and RNS generation during alachlor conversion

The mechanisms of alachlor toxicity may be correlated with its ability to induce oxidative stress by an enhanced production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which in turn may lead to biological macromolecules damage (Rattanawong et al., 2015). However, little is known about the ROS and RNS action towards alachlor-degrading microorganisms. Literature data indicate that the ROS and RNS system may be involved in microbial transformation of xenobiotics, which was postulated in the degradation of 4-n-nonylphenol by *Metarhizium robertsii* (Szewczyk et al., 2014). Therefore, the presence of superoxide anion and nitric oxide in *T. koningii* alachlor degrading mycelia was measured. The quantities of RNS and ROS to the 24-h and 72-h fungal mycelia exposed to the herbicide are presented in Table 2. Two-way ANOVA analysis indicated that interaction between effect of fungal cultures and time significant influenced on the

Table 2
Relative amounts [%] of $O_2^{\bullet-}$ and NO$^{\bullet}$ generated in *T. koningii* mycelium during alachlor transformation.

Parameter	24 h		72 h	
	Control	Alachlor	Control	Alachlor
$O_2^{\bullet-}$ [%]	3.65 ± 0.50	29.54 ± 3.60*	0.10 ± 0.00	0.19 ± 0.01
NO. [%]	0.31 ± 0.01	1.60 ± 0.02*	0.00 ± 0.00	0.01 ± 0.00

* Indicates values that differ significantly from the control at $P < 0.001$, each value is the mean ± SD.

formation of $O_2^{\bullet-}$ and NO$^{\bullet}$ ($F = 22248.26$, $P < 0.001$; $F = 22248.2$, $P < 0.001$, respectively). The addition of alachlor in 24-h cultures caused an 8-fold increase in the level of $O_2^{\bullet-}$ and a 5-fold increase in the level of NO$^{\bullet}$ compared to control. However in 72-h alachlor-supplemented samples, a significant decrease ($P < 0.05$) in the formation of free radicals was noted, which was correlated with an efficient decrease in the content of alachlor (Fig. 1), as well as also with detoxification process (Table 1). Such a phenomenon was not observerd during the herbicide biotransformation by *P. marquandii* in liquid batches. Despite the effective elimination of alachlor *by P. marquandii*, there was no decrease in herbicide toxicity and the content of ROS in the culture remained at a constant level (Słaba et al., 2013c, 2015).

3.3. Laccase production during alachlor elimination in the presence of different concentrations of copper

Laccases are enzymes widely distributed in plants, bacteria, or fungi. These enzymes are polyphenolic oxidases belonging to a large family of enzymes, referred to as multicopper oxidases (Chen et al., 2015b). Some *Trichoderma* species: *T. atroviride* (Chakroun et al., 2010), *T. aureoviridae* (Khambhaty et al., 2015), *T. harzianum* (Sadhasivam et al., 2008) and also *T. koningii* (Wang et al., 2012) have the ability to produce laccase. The influence of alachlor on laccase activity and contribution of this enzyme to the elimination of chloroacetanilides has not been studied so far. Enzyme activity in *T. koningii* cultures supplied with alachlor was measured every 24 h for 7 days as its ability to ABTS oxidize by laccase or another multicopper oxidase. At this stage of work it was not possible to state clearly whether this enzyme was certainly laccase. The formation of the enzyme in the growth medium in the absence of copper ions was negligible (0.02–0.42 U L^{-1}), which suggests that their presence in culture medium is strictly necessary for the production of the enzyme by *T. koningii*. Our results indicated that the addition of Cu^{2+} (Fig. 3**a**) into the culture medium significant stimulated laccase activity, compared to control ($P < 0.05$). The production of laccase by *T. koningii* occurred during the initial 24 h and reached its maximum at 96 h (7.7–13.06 U L^{-1}) for cultures supplemented with various concentrations of copper. In addition, a simultaneous application of copper ions and alachlor also resulted in the enhancement of laccase activity (Fig. 3**b**) (maximum 4.2–9.58 U L^{-1} for different concentrations of copper at 72–96 h). In cultures older than 96 h, the enzyme activity decreased and after 168 h, it reached a very low level compared with control cultures not supplemented with Cu^{2+}, whereas in the presence of alachlor, minor laccase activity was detected until 168 h. Two-way ANOVA showed that the interaction between samples with different concentrations of copper and with or without alachlor and time significantly influenced on laccase activity ($F = 905.67$, $P < 0.001$; $F = 1538.2$, $P < 0.001$, respectively). The optimal copper concentration for laccase synthesis was found to be 5.0 and 7.5 mM. Stimulation of laccase activity by the addition of copper into the culture medium is well documented and may result from the filling of type-2 copper binding sites with copper ions (Chakroun et al., 2010; Khambhaty et al., 2015). However, some heavy metals such as copper or vanadium may be involved in the oxidative production of radicals by Fenton or Haber-Weiss reactions, which may enhance nonspecific

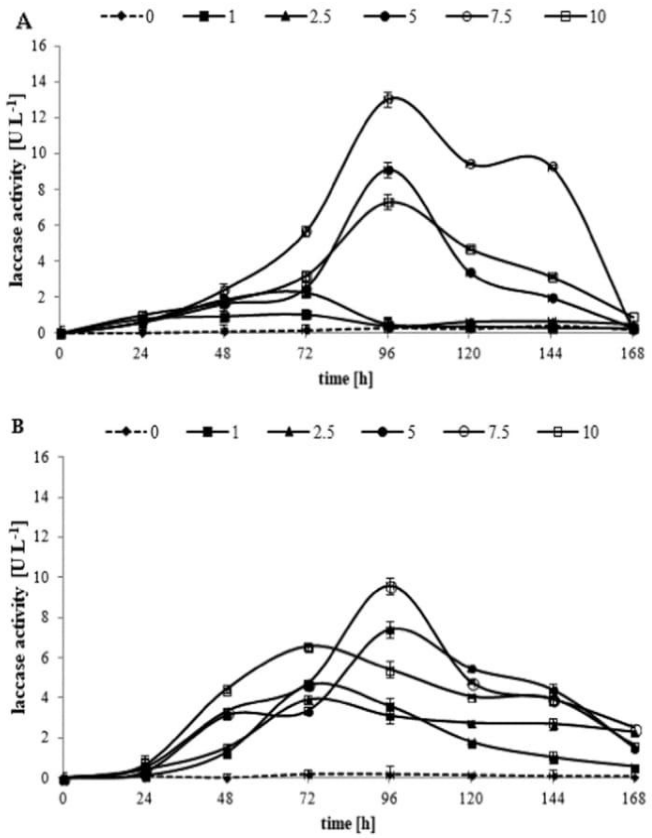

Fig. 3. Laccase activity in supernatants collected from fungal cultures supplemented with copper ions (1–10 mM) (**A**) and simultaneously with alachlor (50 mg L^{-1}) and copper ions (1–10 mM) (**B**). Data are presented as mean ± SD (n = 3).

enzyme activities (Liu et al., 2017). Laccase activity may also be stimulated by oxidative stress (Ceci et al., 2018). The toxicity of alachlor (Rattanawong et al., 2015; Słaba et al., 2015) and copper (Słaba et al., 2013b) might be associated with the induction of oxidative stress in fungal cells. Enhanced radicals production was also detected in alachlor treated *Trichoderma* cultures within the first 24 h of incubation (Table 2). Laccase activity stimulation by different xenobiotics is well documented. Mougin et al. (2002) reported an increased laccase activity in a *Tremetes versicolor* culture supplemented with different compounds of industrial origin (e.g., nonylphenol, aniline, diquox, or 9-fluorenone). An increased activity of this enzyme during the biodegradation of bisphenol A and diclofenac by *T. versicolor* was noted by Yang et al. (2013). The greatest effect on the enzyme activity in a liquid culture of *T. koningii* was detected after 3–4 days of treatment. Similar results were obtained by Sadhasivam et al. (2008), who reported that the onset of laccase activity in *Trichoderma harzianum* occurred at 48 h and achieved the maximum at 96 h of incubation. Subsequently, the enzyme production decreased. In the case of *Trichoderma atroviride*, the enzyme activity was noted only after 5 days of culture (Chakroun et al., 2010).

3.4. Effect of metabolic inhibitors on alachlor elimination by T. koningii

Studies on fungal laccases and cytochrome P450 oxidases have revealed a potential role of these oxidative enzymes in the metabolism of different xenobiotics, for example alachlor (Chirnside et al., 2011; Pothuluri et al., 1993), bisphenol A (Yang et al., 2013), 2,4-dichlorophenoxyacetic acid (2,4-D) (Nykiel-Szymańska et al., 2018), dibutyltin (Siewiera et al., 2017), diethyltoluamide (Tran et al., 2013), or malachite green (Jasińska et al., 2015). Therefore, we investigated whether *T. koningii* was able to transform alachlor in the presence of

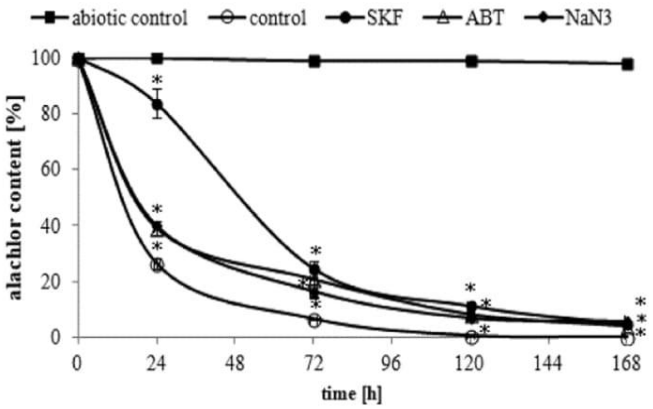

Fig. 4. Degradation of alachlor by *Trichoderma koningii* strain in the presence of metabolic inhibitors. * Indicates values that differ significantly from the control at $P < 0.001$, each value is the mean ± SD (n = 3).

inhibitors of laccase and CYP450. Degradation of alachlor by the *T. koningii* strain in the presence of a putative inhibitor of laccase (0.1 mM sodium azide) was monitored. Competitive inhibitors of CYP450, such as aminobenzotriazole and proadifen were also used (Fig. 4**).** An initial concentration of the inhibitors was individually chosen for *T. koningii* as the highest dose, which did not inhibit the fungal growth by more than 15%.

The sensitivity of laccase to several supposed enzyme inhibitors such as sodium dodecyl sulfate (SDS), NaN_3, and ethylenediaminetetraacetic acid (EDTA) was tested (Table S2). Among the tested compounds, NaN_3 was found to be the most effective laccase inhibitor. Literature also indicates that NaN_3 is an effective inhibitor of fungal laccase (Chakroun et al., 2010; Sadhasivam et al., 2008). The enzyme activity was completely inhibited at a concentration as low as 0.1 mM. The addition of 0.1 mM NaN_3 to the fungal culture resulted in a small but statistically significant drop in the rate of alachlor decomposition, which indicates the contribution of this enzyme in the elimination of chloroacetanilide (Fig. 4).

The addition of CYP450 inhibitors to the culture medium of *T. koningii* led to a significant inhibition of alachlor biodegradation ($P < 0.05$) during the first 24 h, when its elimination was most effective. Two-way ANOVA analysis showed significant effect of treatment of CYP450 inhibitors ($F = 359.77$, $P < 0.001$), incubation time ($F = 1627.24$, $P < 0.001$) and interaction among this factors ($F = 124.08$, $P < 0.001$) on alachlor degradation by tested fungus. After 24 h in the fungus culture with 0.1 mM proadifen, alachlor removal was about 60% lower, and subsequently, the influence of the inhibitor on the degradation rate was found to be weaker but statistical significant ($P < 0.05$). In contrast, in the sample with the addition of 0.25 mM 1-aminobenzotriazole, the elimination of the herbicide was observed lower by 5–15%. These results show a significant role played by CYP450 in the initial stage of alachlor biotransformation (24 h), but later the process of alachlor removal was not dependent on CYP450 activity. Earlier studies showed different significance of CYP450 in fungal chloroacetanilides metabolism. The CYP450 system was found to be strongly involved in the utilization of alachlor by *C. elegans* (Pothuluri et al., 1993). However, according to Słaba et al. (2013c), CYP450 has a negligible role in the elimination of alachlor by *P. marquandii.* Throughout the culture period, the herbicide removal in the presence of inhibitors was about 20% lower than without inhibitors, but after 7 days of incubation, the differences between the samples with and without inhibitors did not exist.

3.5. The influence of copper on herbicide degradation

Contamination of the environment with heavy metals and chlorinated organic compounds (e.g., alachlor, 2,4-D, 4-chlorophenol, DDT,

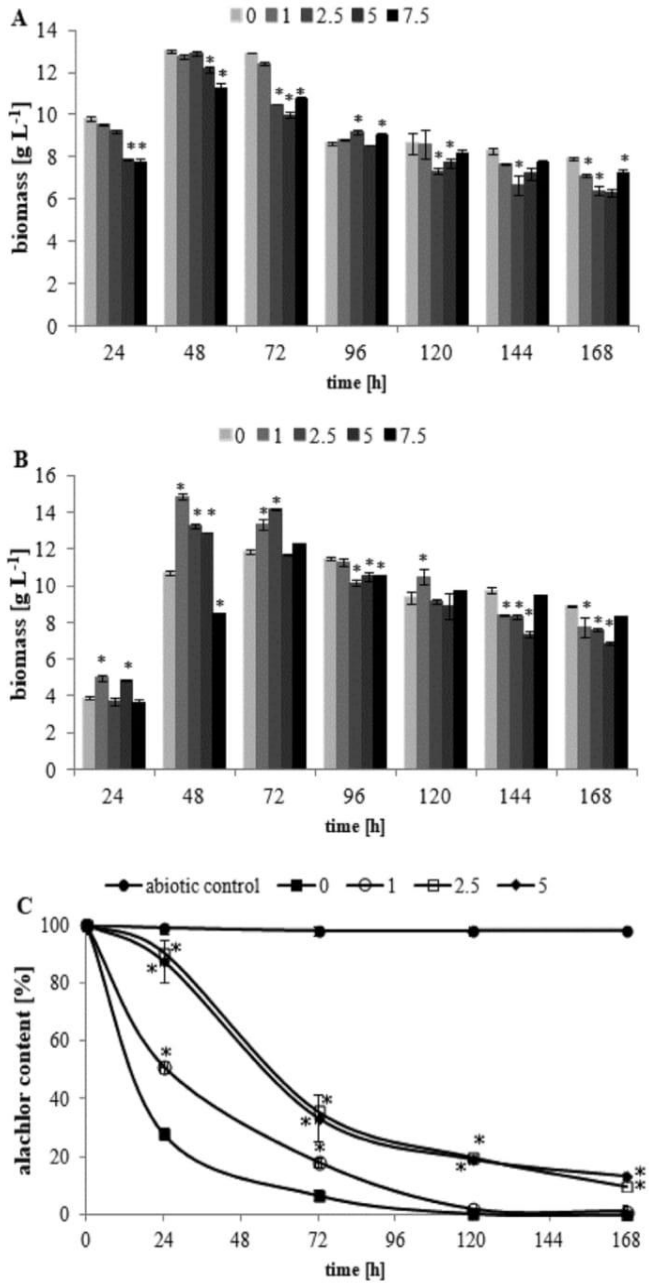

Fig. 5. *Trichoderma koningii* growth and alachlor degradation in the presence of copper ions. Fungal growth in the presence of 1.0–7.5 mM Cu^{2+} (**A**). Simultaneous effect of 50 mg L^{-1} alachlor and copper ions (1.0–7.5 mM) on fungal growth (**B**). Alachlor elimination in the presence of copper ions (1.0–5.0 mM) (**C**). * Indicates values that differ significantly from the control at $P < 0.001$, each value is the mean ± SD (n = 3).

and trichloroaniline) is a serious problem in industrial and agricultural areas (Olaniran et al., 2009). Heavy metals may inhibit the growth of microorganisms involved in the degradation of organic pollutants and affect the rate of biodegradation by inhibiting enzymes engaged in the metabolism of these compounds (Chen et al., 2015a; Liu et al., 2017).

The growth intensity of *T. koningii* in the presence of copper and simultaneous presence of copper and alachlor was tested (Fig. 5**a,b**). The tested strain was characterized by high tolerance to the presence of copper ions in the culture in the range of concentrations 1–7.5 mM (63.5–476.25 mg L^{-1}) (Fig. 5**a**). The fungal biomass growth in cultures supplemented with 1.0 and 2.5 mM copper was comparable to the control, whereas at higher concentrations (5.0 mM and 7.5), only 20% inhibition of growth was noted. Two-way ANOVA revealed no significant effect of different concentrations of heavy metal ($F = 0.97$, $P = 0.45$) and time ($F = 1.02$, $P = 0.41$) on growth of *T. koningii* as well as no interaction between this factors ($F = 0.99$, $P = 0.49$) was verified. Strong growth inhibition in alachlor- and copper-treated cultures, observed during the initial 24 h of incubation, was caused by alachlor (Figs. 1, 5**a**, 5**b**). Cu^{2+} did not deteriorate the growth of *T. koningii* supplemented with the herbicide. Moreover, the metal at concentrations of 1.0–5.0 mM, stimulated biomass production in 48 h alachlor-stressed cultures. Again, two-way ANOVA showed no significant effects due to the treatment ($F = 0.99$, $P = 0.43$), time ($F = 1.02$, $P = 0.43$) or interaction effect ($F = 0.99$, $P = 0.5$). Our results indicate that the filamentous fungus *T. koningii* survived at the copper concentrations which might be toxic to other *Trichoderma* sp. The growth of *T. asperellum* strongly decreased with increasing the initial concentration of copper. After 10 days of incubation in fungal cultures supplemented with 300 mg L^{-1}, more than 90% inhibition of growth was observed (Iskandar et al., 2011). Lòpez and Vázquez (2003) reported that *T. atroviride* was also characterized by high tolerance to copper at concentrations of 0.25–300 mg L^{-1}. In this range, only ca. 10% deterioration in growth was observed. However, at 350 mg L^{-1} of metal concentration, the growth of *T. atroviride* severely decreased (ca. 80%). In addition, Anand et al. (2006) reported that there was no growth of *T. viride* at 300 mg L^{-1} of Cu^{2+}. Modification in metal tolerance might be due to the presence of one or more types of tolerance strategies or resistance mechanisms demonstrated by different microorganisms (Mohammadian et al., 2017).

The results indicate that at 24 h, alachlor degradation in the presence of 1.0 mM copper was ca. 20% lower in comparison to cultures not supplemented with Cu^{2+}. The differences between the culture with and without copper ions did not exist since 120 h (Fig. 5**c**). At higher concentrations of copper (2.5 and 5.0 mM), alachlor biotransformation was significantly slower (ca. 30–60%), but finally it caused only 20% deterioration in the pesticide degradation ($P < 0.05$). Two-way ANOVA demonstrated significant effect of different concentrations of copper ($F = 30657.5$, $P < 0.001$), incubation time ($F = 10245$, $P < 0.001$) as well as their interaction effect ($F = 1315.8$, $P < 0.001$) on alachlor elimination by *T. koningii*. Some selected microorganisms can eliminate xenobiotics in the presence of toxic metals (Ceci et al., 2018; Hong et al., 2010; Olaniran et al., 2009; Słaba et al., 2009; Springael et al., 1993). Similarly, elimination of alachlor was not inhibited in the culture of *P. marquandii* with the addition of 1.0 mM zinc. Nevertheless, a higher metal content (5.0 and 7.5 mM) in the culture medium limited alachlor mitigation by 30–50% (Słaba et al., 2009). Synergic action of heavy metals and xenobiotics might activate specific metabolic pathways and nonspecific oxidoreductive enzymes such as laccases (Ceci et al., 2018). Inhibition of biodegradation of 2,4-D in copper co-contaminated systems has been reported by Springael et al. (1993). Iwinski et al. (2017) studied the influence of copper on bacterial degradation of microcystin, a toxin produced by freshwater bacteria. The results indicated that copper even at low concentrations (1.0–5.0 mg L^{-1}) inhibited the elimination of the toxin by as much as 36–70%. According to Olaniran et al. (2009), the presence of lead and mercury causes 24% reduction in the degradation of 1,2-dichloroethane in soil.

Filamentous fungi, isolated from contaminated environments, are often characterized by high tolerance to heavy metals and have been proposed as potential bioagents for metal recovery processes (Chen et al., 2015a; Wang and Wang, 2013). We studied the uptake of copper to evaluate the ability of *T. koningii* to eliminate alachlor and simultaneously remove copper from the culture containing both toxic compounds. The results of Cu^{2+} uptake by the IM 0956 strain in the presence of alachlor are presented in Table S3. *Trichoderma* mycelia originating from 72-h and 120-h cultures, whose growth was not inhibited by increasing concentrations of copper ions (1.0–7.5 mM), bound 0.9–6.15 mg and 0.97–7.76 of copper g^{-1} in 1 g of dry mycelium, respectively. The metal uptake in mycelia increased proportionally to the initial metal concentration. *T. koningii* has already been mentioned as a cadmium-tolerant strain (Massaccesi et al., 2002), but

this is the first report on copper tolerance and removal by this species. Another species of *Trichoderma*, *T. atroviride*, isolated from sewage sludge, was able to remove 1.2 mg copper g^{-1} dry weight at 50 mg L^{-1} of Cu^{2+} (López and Vázquez, 2003). In the culture of *T. asperellum*, the maximum uptake of Cu^{2+} occurred at 200 mg L^{-1} concentration with a value of 12.809 mg g^{-1} (Iskandar et al., 2011). A similar content of copper was noted in the mycelia of *T. koningii*, originating from the stationary phase of growth (7.76 mg of copper in 1 g of dry mycelium).

4. Conclusion

This study demonstrates that *T. koningii* can effectively eliminate alachlor even in the presence of copper ions. The results indicate that the herbicide biotransformation is a complex process involving CYP450 monooxygenase(s) and laccase. Moreover, the study shows that xenobiotic biodegradation is accompanied by the detoxification of alachlor. Stimulation of laccase activity in *T. koningii* cultures supplemented with copper and alachlor could enhance the degradation potential of the tested strain. This observation may be helpful for investigation of bioremediation areas co-contaminated with copper and chloroacetanilide herbicides using the *T. koningii* strain.

Acknowledgements

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Appendix A. Supporting information

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Supplementary

Table S1. Laccase activity in supernatants from *Trichoderma* spp. cultures with or without 50 mg L^{-1} alachlor collected from 72 h of incubation. The results are the average of triplicate analysis.

Fungal cultures	Enzyme activity [U L^{-1}]	
	Biotic control	Sample with alachlor
T. koningii IM 0956	0.16	0.2
T. atroviride IM QF10	0.06	0.05
T. hamatum IM I-1	0	0
T. harzianum IM 0961	0	0
T. harzianum KKP 534	0	0
T. citrinoviride IM 6325	0	0
T. virens DSM 1963	0	0
T. viride KKP 792	0	0

Table S2. Effect of inhibitors on laccase activity in supernatants from *Trichoderma koningii* cultures supplemented with 1 mM copper ions or simultaneously with 1 mM copper ions and alachlor (50 mg L^{-1}) collected from 72 h of incubation. The results are the average of triplicate analysis.

Compound name	Concentration [mM]	Inhibition [%]			
		control	1 mM Cu^{2+}	alachlor	1 mM Cu^{2+} + alachlor
EDTA	0.1	75	60	87	64
	1	89	85	92	72
NaN_3	0.1	100	100	100	100
	1	100	100	100	100
SDS	0.1	65	48	60	37
	1	99	89	100	92

Table S3. Copper uptake by *Trichoderma koningii* mycelium during alachlor transformation. Data are presented as mean ± SD (n=3).

Incubation period (h)	**Copper content in mycelium [mg g^{-1} of dry weight]**			
	Cu^{2+}concentration (mM)			
	1.0	**2.5**	**5.0**	**7.5**
72	0.9±0.2	3.2±0.6	4.92±0.02	6.15±0.01
120	0.97±0.2	2.3±0.3	7.76±0.6	5.11±0.01

Artykuł naukowy nr 3

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Assessment of oxidative stress and phospholipids alterations in chloroacetanilides-degrading *Trichoderma* spp

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ABSTRACT

To investigate the induction of oxidative stress and antioxidant response in the chloroacetanilides-degrading *Trichoderma* spp. under alachlor and metolachlor exposure, a comparative analysis using popular biomarkers was employed. An increased intracellular level of reactive oxygen species (ROS; especially superoxide anion [$O_2^{\cdot -}$]) as well as products of lipid and protein oxidation after 24 h incubation with the herbicides confirmed chloroacetanilide-induced oxidative stress in tested *Trichoderma* strains. However, the considerable decline in the ROS levels and the carbonyl group content (biomarkers of protein peroxidation) in a time-dependent manner and changes in the antioxidant enzyme activities indicated an active response against chloroacetanilide-induced oxidative stress and the mechanism of tolerance in tested fungi. Moreover, the tested herbicides clearly modified the phospholipids (PLs) content in *Trichoderma* spp. in the stationary phase of growth, which was manifested through the difference in phosphatidic acid (PA), phosphatidylethanolamine (PE) and phosphatidylcholines (PC) levels. Despite enhanced lipid peroxidation and changes in PLs in most tested fungi, only a slight modification in membrane integrity of *Trichoderma* spp. under chloroacetanilides exposure was noted. The obtained results suggest that the alterations in the antioxidant system and the PLs profile of *Trichoderma* spp. might be useful biomarkers of chloroacetanilide-induced oxidative stress.

1. Introduction

Alachlor (2-chloro-N-2,6-diethylphenyl-N-[methoxymethyl]acetamide) and metolachlor (2-chloro-N-[2-ethyl-6-methylphenyl]-N-[2-methoxy-1-methylethyl]acetamide) are pre- and post-emergent herbicides commonly used in the control of annual grasses and weeds in corn, cotton, rapeseed, rice, and soybean fields (Huang et al., 2017). The major issue with the constant and uncontrolled use of these herbicides is the contamination of environment and toxic effect on non-target organisms (Gil et al., 2017). Alachlor and metolachlor are classified as endocrine-disrupting compounds (Mnif et al., 2011) and probable human carcinogens (US EPA, 2017). Nevertheless, alachlor is definitely more toxic to mammals (rat, mouse, and rabbit) and fish than metolachlor, therefore, since 2008, the use of alachlor has been banned in the European Union and Canada (Bergman et al., 2012). The harmful effects of the herbicides may also be related to triggering the formation of reactive oxygen species (ROS) or antioxidant system depletion, leading to an imbalance between oxidants and antioxidants and thus cause oxidative damage to biological macromolecules (lipids, proteins or DNA) (Jiang et al., 2016; Sies et al., 2017). Although the induction of oxidative stress under chloroacetanilide herbicides exposure and the antioxidant system response were reported, the data were mostly related to plants (Singh et al., 2017; Štajner et al., 2004), green microalgae (Maronić et al., 2018), fish (Yi et al., 2007), or rat tissues (Burman et al., 2003). However, there is still a special interest in the oxidative stress response in microorganisms, especially in those that can degrade these compounds. The effects of selected chloroacetanilides (alachlor, acetochlor, and metolachlor) on the antioxidant system of yeast *Saccharomyces cerevisiae* (Rattanawong et al., 2015), filamentous fungus *Paecilomyces marquandii* (Słaba et al., 2015), and soil bacteria (Martins et al., 2011) have been investigated, but the mechanism of oxidative disturbance and microbial response in chloroacetanilide-transforming microorganisms is not fully understood.

Previous studies showed that the filamentous fungus *Trichoderma koningii* IM 0956 could transform alachlor effectively, even in the presence of copper. Moreover, the elimination of the tested chloroacetanilide resulted in an almost complete reduction in the toxicity to *Artemia franciscana* larvae. The pathway of alachlor conversion by tested fungus involved mainly dechlorination and hydroxylation reactions (Nykiel-Szymańska et al., 2018).

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The aim of this study was to evaluate oxidative stress induction and antioxidant response during alachlor and metolachlor elimination by eight *Trichoderma* strains. Specifically, oxidative stress biomarkers such as intracellular ROS generation, lipids, and protein peroxidation were revealed. The antioxidant defense of the tested fungal strains was determined by measuring the activity of catalase (CAT) and superoxide dismutase (SOD). Additionally, we focused on the possible modification of the *Trichoderma* spp. lipid profile and membrane permeability under the tested chloroacetanilides.

2. Materials and methods

2.1. Chemicals

Alachlor and metolachlor (PESTANAL, analytical standard [99.2%]), nitrotetrazolium blue chloride (NBT), sodium azide (NaN_3), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), 2,4-dinitrophenylhydrazine (DNPH), bovine serum albumin (BSA), Bradford reagent, ethylenediaminetetraacetic acid (EDTA), polyvinylpyrrolidone, methionine, riboflavin, streptomycin sulfate, butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), trichloroacetic acid (TCA) and malondialdehyde (MDA) were obtained from Sigma-Aldrich (Poznan, Poland). Phospholipids (PLs) standards were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Ethyl acetate was obtained from POCh (Gliwice, Poland), and high-purity solvents used during the sample preparation for high-pressure liquid chromatography were obtained from Sigma-Aldrich (Poznan, Poland) and POCh (Gliwice, Poland).

2.2. Microorganisms and growth conditions

Five *Trichoderma* strains such as: *T. atroviride* IM QF10, *T. hamatum* IM I-1, *T. harzianum* IM 0961, *T. koningii* IM 0956, and *T. citrinoviride* IM 6325 were obtained from the Microorganisms Collection of the Department of Industrial Microbiology and Biotechnology, University of Lodz (Lodz, Poland). *T. harzianum* KKP 534 and *T. viride* KKP 792 strains were deposited in the Collection of the Industrial Microorganisms of the Institute of Agricultural and Food Industry (IAFB) (Warsaw, Poland). *T. virens* DSM 1963 was acquired from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The ability of *T. koningii* IM 0956 to eliminate alachlor has been previously reported by Nykiel-Szymańska et al. (2018).

The spores of tested *Trichoderma* spp. originating from 7-day-old cultures were used to inoculate 20 mL mineral medium with glucose (2%) and yeast extracts (1%). The initial preculture was cultivated under conditions described previously (Nykiel-Szymańska et al., 2018). Two milliliters of the homogenous preculture was introduced into 18 mL of medium supplemented with alachlor or metolachlor at a concentration of 50 mg L^{-1} (stock solution 10 mg mL^{-1}) or without chloroacetanilides in the control cultures. The abiotic controls of the tested herbicides (uninoculated) were also prepared. All cultures were incubated at 28 °C on a rotary shaker (160 rpm). Then, the *Trichoderma* spp. mycelia samples were washed and separated for analyses. The samples from the exponential (24 h) and stationary (72 h) phases of growth for the control and chloroacetanilides-treated mycelium were used in the experiments.

The *Trichoderma* spp. mycelia were separated by filtration with a vacuum pump and then dried at 100 °C, to reach a constant weight for dry biomass determination.

2.3. Extraction and quantification of chloroacetanilides

The extraction of chloroacetanilide herbicides from *Trichoderma* spp. cultures was performed according to the procedure described by Nykiel-Szymańska et al. (2018). The quantitative analysis of alachlor and metolachlor in the fungal cultures was carried out by the chromatographic method (LC Agilent 1200 coupled with an Sciex 3200 mass spectrometer) described in our previous study (Nykiel-Szymańska et al., 2018). The detection of the herbicides was performed using tandem mass spectrometry detector working in the multiple reaction monitoring (MRM) positive ionization mode. The m/z values of monitored MRM pairs for alachlor were 270.1–238.2 and 270.1–162.3 at a retention time of 1.61 min. Moreover, m/z values of the MRM pairs for metolachlor were 284.2–252.2 and 284.2–176.2 at a retention time of 1.59 min.

2.4. Measurement of intracellular peroxynitrate anion/hydroxyl radical anion

The measurement of the total intracellular levels of peroxynitrate anion/hydroxyl radical anion ($HO\cdot/ONOO^-$) was carried out using 2′,7′–dichlorodihydrofluorescein diacetate (H_2DCFDA) assay described by Liu et al. (2010), with some modifications. Briefly, 1 mL of *Trichoderma* spp. cultures was centrifuged at 8000 × *g* for 10 min. Next, the supernatant was removed, and the biomass was suspended in 1 mL of phosphate-buffered saline (PBS) containing 50 mM H_2DCFDA (previously dissolved in dimethyl sulfoxide, [DMSO]). The samples were incubated for 10 min under the dark at room temperature. Then, the mycelium was washed three times with PBS and placed on a microscope slide. The images were recorded using a confocal laser scanning microscope (LSM510 Meta, Zeiss) combined with an Axiovert 200 M (Zeiss, Germany) inverted fluorescence microscope equipped with a Plan Apochromat objective (63/1.25 oil). The samples were excited with a 488 argon laser line, and the emission spectrum was recorded using a 530 nm bandpass filter. The same laser line was used for Nomarski differential interference contrast microscopy. The results were expressed as a percentage of the green fluorescence area compared to the total hyphal area.

2.5. Measurement of intracellular superoxide anion

For the measurement of superoxide anion ($O_2\cdot^-$) in *Trichoderma* spp. hyphae, the method described in the study by Trujillo et al. (2004) was used. Briefly, 1 mL of fungal culture was centrifuged at 8000 × *g* for 10 min. Next, the supernatant was removed and the biomass was suspended in 1 mL solution containing 0.1% NBT and 10 mM NaN_3 in sodium phosphate buffer (PBS) of pH 7.8. The samples were incubated for approximately 60 min under the dark at room temperature. Next, the biomass was examined under a confocal laser scanning microscope LSM510 Meta (Zeiss). The results were expressed as a percentage of the blue area compared to the total hyphal area.

2.6. Enzyme activity determination

The enzyme extracts were prepared according to the method described in the study by Słaba et al. (2015). Briefly, fresh *Trichoderma* spp. mycelium (1 g) was homogenized (1:10 w/v) in a frozen mortar with the addition of 50 mM sodium phosphate buffer pH 7 containing 1% polyvinylpyrrolidone, 10 mM sodium ascorbate and 1 mM EDTA. After centrifugation (15,000 × *g*, 10 min, 4 °C) the obtained supernatant was used for the measurements of enzyme activity. Catalase (CAT) activity was determined spectrophotometrically by monitoring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm (Dhindsa et al., 1981). The reaction mixture for CAT activity contained 50 mM sodium phosphate buffer pH 7, 1% H_2O_2 and enzyme extract. Superoxide dismutase activity was measured at 540 nm by the nitrotetrazolium blue chloride (NBT) reduction method described by Beauchamp and Fridovich (1971). The assay mixture consisted of 50 mM sodium phosphate buffer pH 7,8, EDTA, NBT, methionine, ryboflavine and the enzyme extract. The protein content was estimated by the Bradford procedure (1976) using BSA as a standard.

2.7. Measurement of protein carbonyl content

Protein oxidative damage was measured spectrophotometrically as protein carbonyl content using the 2,4-dinitrophenylhydrazine (DNPH) assay described by Levine et al. (1994), with some modifications. Fresh fungal biomass (0.5–1 g) was homogenized in an ice-cold mortar using 50 mM PBS (pH 6.7) containing 1 mM EDTA. After centrifugation (10,000 × *g*, 15 min, 4 °C), the obtained supernatant was used in the protein carbonyl content assay. First, 0.2 mL of the above-mentioned supernatant was added to 0.8 mL of 10 mM DNPH (dissolved in 2.5 mM HCl) and allowed to remain for 1 h at room temperature. In parallel, control samples were prepared by the addition of 2.5 mM HCl instead of DNPH. All samples were vortexed every 15 min during the incubation. After incubation, 1 mL of 20% trichloroacetic acid (TCA) was added to each sample, left for 5 min and centrifuged (10,000 × *g*, 15 min, 4 °C). Next, the pellets were resuspended in 1 mL of 10% TCA and incubated for 5 min. After centrifugation, the supernatant was discarded, and the pellet was washed three times with 1 mL of ethanol/ethyl acetate mixture (1:1) with resuspension followed by centrifugation. The washed pellets were dissolved in 0.5 mL 6 M guanidine hydrochloride, and the insoluble material was removed by centrifugation. The sample absorbance was measured at 370 nm, and by using extinction coefficient for DNPH (22 mM^{-1} cm^{-1}), the concentration of the carbonyls was calculated as nanomoles of protein carbonyls per mg of protein. The protein amount was determined on the basis of the absorbance at 280 nm.

2.8. Lipid peroxidation assay

Lipid oxidation damage was measured spectrophotometrically as the content of thiobarbituric acid-reactive substances (TBARS) using Jo and Ahn's assay (1998), but slightly modified. Fresh wet biomass (0.50 g) was homogenized with a ball mill (MM 400, Retsch) using 9 mL of deionized water containing 0.05 mL of 7.2% BHT. Next, 1 mL of the fungal homogenate was transferred to a Falcon tube containing 2 mL TBA-TCA solution (20 mM TBA in 15% TCA). Then, the mixture was vortexed and boiled in a water bath for 30 min. After cooling, the samples were centrifuged at 2000 × *g* for 15 min. The absorbance of supernatants was measured at 531 nm and the value of nonspecific absorption was subtracted at 600 nm.

2.9. Determination of phospholipids

Phospholipids (PLs) of tested *Trichoderma* spp. were extracted according to the procedure described in the study by Bernat et al. (2018). Fresh fungal biomass (0.10 g) was transferred into an Eppendorf tube (1.50 mL) containing methanol (0.66 mL), chloroform (0.33 mL) and also glass beads. The samples were homogenized for 2 min using a ball mill (FastPrep-24, MP Biomedicals). Next, 0.20 mL of 0.9% NaCl was added to the obtained mixture to facilitate separation into two layers. The quantitative and qualitative analyses of PLs were performed by LC-MS/MS technique (LC Agilent 1200 coupled with an Sciex 4500 tandem mass spectrometer) according to the method described by Bernat et al. (2018). Chromatographic separation was performed on a Kinetex C18 column (50 mm × 2.1 mm, particle size: 5 µm; Phenomenex, Torrance, CA, USA) heated to 40 °C with a flow rate of 0.5 mL min^{-1}. The mobile phases were water (A) and methanol (B) both containing 5 mM ammonium formate. The solvent gradient started at 70% B, after 0.25 min increased to 95% B during 1 min, and remained at 95% B for 7 min before returning to 70% B over 2 min. The mass spectrometer ion source worked in the negative mode, spray voltage −4.500 V, curtain gas 25, nebulizer gas 60, auxiliary gas 50, and temperature 600 °C. The data analysis was performed with Analyst™ v1.6.2 software (Sciex, Framingham, MA, USA).

2.10. Statistical analyses

All samples were prepared in triplicate, and the experiments were repeated two separate times. The samples variability was calculated as a standard deviation (± SD). Data were analyzed by the two-and three-way analysis of variance (ANOVA) and means were compared using Tukey's test ($P < 0.05$). Statistical analyses were performed using the software STATISTICA version 13.1 (StatSoft).

3. Results and discussion

3.1. Effects of chloroacetanilides on growth and intracellular ROS generation

Different strains of *Trichoderma*: *T. atroviride* IM QF10, IM I-1, *T. harzianum* IM 0961, *T. harzianum* KKP 534, *T. citrinoviride* IM 6325, *T. viride* KKP 792, and *T. virens* DSM 1963 were able to eliminate alachlor similarly to *T. koningii* IM 0956, which the growth in the presence of alachlor and its ability to transform this herbicide have been described previously (Nykiel-Szymańska et al., 2018). Additionally, metolachlor degradation by the above-mentioned *Trichoderma* spp. was also studied. The growth pattern of the *Trichoderma* strains differed after the addition of the examined chloroacetanilide herbicides, and it was difficult to determine which of them was more harmful to the tested microorganisms. In most cases chloroacetanilides inhibited fungal growth during the first 24 h of incubation (Fig. 1). These chloroacetanilides-degrading fungi were characterized by different rates of herbicide transformation. Alachlor was more effectively removed from *Trichoderma* cultures than metolachlor. After 72 h alachlor removal reached 80–98%, whereas metolachlor elimination ranged 15–65% (Fig. 2). The highest reduction in the metolachlor content was noted after 120 h of incubation (69–84%), but at this point of time, the activity of antioxidant enzymes was negligible (data not shown). Moreover, our former study (Nykiel-Szymańska et al., 2018) indicated reactive oxygen species alleviation during alachlor biotransformation by *T. koningii* IM 0956, which encouraged us to continue and expand investigations about oxidative disturbance and microbial response in chloroacetanilide transforming fungi from the genus *Trichoderma*. The intracellular ROS level in *Trichoderma* spp. mycelia after chloroacetanilides exposure was inspected at 3, 6 and 12 h of incubation, but we did not observe any changes between the biotic controls and the tested samples (data not shown). Chloroacetanilides oxidative action was noted after 24 h. Therefore, the evaluation of ROS level in the tested fungi under chloroacetanilides exposure was conducted both in the exponential (24 h) and early stationary (72 h) phases of growth.

Literature data indicated that the mechanisms of toxicity of chloroacetanilide herbicides could be associated with the enhanced production of intracellular ROS (Jiang et al., 2016). Moreover, reactive molecules can be produced during the early step of microbial transformation of this type of herbicides, which involves the reaction of dechlorination (Xu et al., 2008). Therefore, the intracellular level of ROS including superoxide anion and hydroxyl radical/peroxynitrate anion in *Trichoderma* spp. mycelia after 50 mg L^{-1} alachlor and metolachlor exposure was determined (Table 1). The H_2DCFDA and NBT assays, used in this study, are one of the basic tools to evaluate cellular oxidative stress by the determination of reactive oxygen species (Bernat et al., 2018; Jambunathan, 2010; Małolepsza and Rózalska, 2005; Maronić et al., 2018; Słaba et al., 2015; Siewiera et al., 2017). H_2DCFDA is a non-specific indicator of oxidative stress, which is commonly used for the determination of the overall ROS level (Bernat et al., 2018; Maronić et al., 2018; Słaba et al., 2015). However, according to Kalyanaraman et al. (2012) and data from the Molecular Probes Handbook (Thermofisher) (Johnson and Spence, 2010), this indicator may be considered among others as a non-specific indicator of the HO ·/$ONOO^-$ level. Our results also suggest this possibility, because the level of intracellular superoxide anion was definitely higher and did not

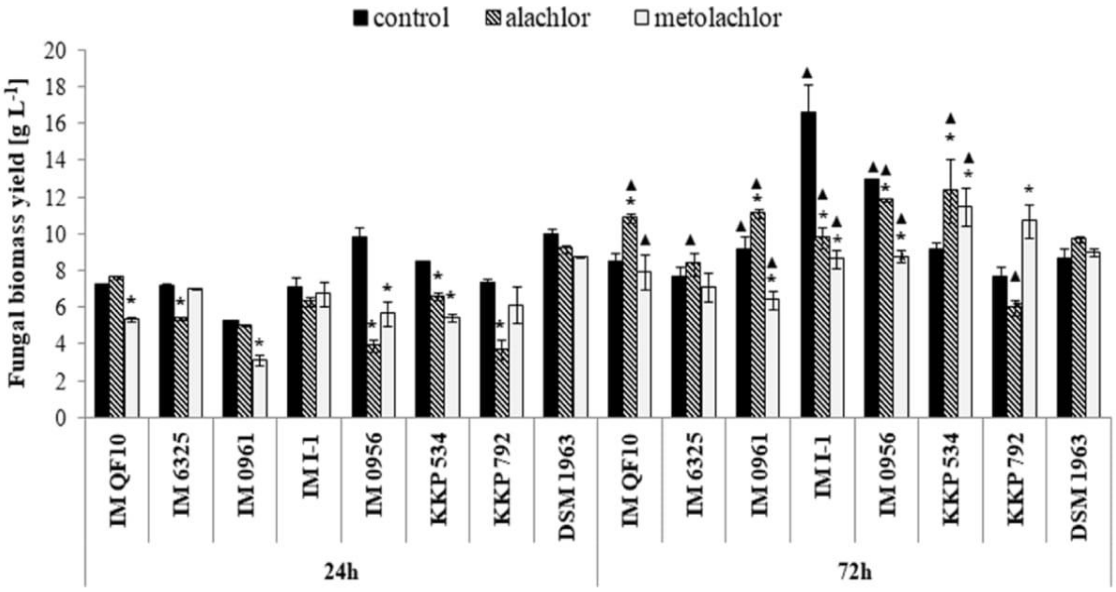

Fig. 1. Growth of the tested *Trichoderma* spp. in the presence of alachlor and metolachlor after 24-and 72-h incubation on mineral medium supplied with glucose (2%) and yeast extract (1%). Data are the mean ± SD ($n = 6$). * indicates the values that differ significantly from the biotic control at $P < 0.001$ and ▲ indicates the values that differ significantly in a time-dependent manner, according to the two-way ANOVA followed by Tukey's HSD test.

reflect the overall ROS level. The images originating from 24-h cultures, when the accumulation of reactive molecules was most intense, are presented in Table S1 and Table S2 in Supplementary Data. The interaction between the herbicides exposure and the growth phase significantly influenced ROS formation in *Trichoderma* spp. mycelia ($F = 268.03$, $P < 0.001$ for $HO\cdot/ONOO^-$ and $F = 187.88$, $P < 0.001$ for $O_2\cdot^-$), which was demonstrated in the two-way ANOVA test. The highest level of the reactive molecules in the other chloroacetanilides-treated fungal samples was detected at 24 h of incubation. However, the tested compounds affected the overproduction of superoxide anion more significantly than the other tested ROS. The addition of alachlor or metolachlor to the cultures of IM QF10, IM 0961, IM 0956, and IM 6325 caused a noticeable, approximately 4–to 8-fold, increase in the level of $O_2\cdot^-$, whereas, for KKP 534, KKP 792, and DSM 1963, the herbicide caused an enormous overproduction of these ROS, approximately 15–to 28-fold, compared to the control. The results presented in Table 1 show that the content of hydroxyl radical/peroxynitrate anion in fungal hyphae treated with chloroacetanilides increased, although in most samples without statistical significance. Nevertheless, these results clearly showed the induction of oxidative stress in *Trichoderma* spp. after exposure to the herbicides. Other studies also reported overproduction of intracellular ROS under chloroacetanilides exposure in *Saccharomyces cerevisiae* (Rattanawong et al., 2015) and *Paecilomyces marquandii* (Słaba et al., 2015). In addition, the generation of reactive oxygen species may be considered as an indicator of chloroacetanilides toxicity in *Trichoderma* spp. As presented in Table 1, after 3 days of incubation with the herbicides, the levels of $O_2\cdot^-$ and $HO\cdot/ONOO^-$ were significantly lower in *Trichoderma* mycelium, which was related to the elimination of the tested chloroacetanilides (Fig. 1). For *T. koningii,* it was also linked with reduction in alachlor toxicity to *A. franciscana* larvae (Nykiel-Szymańska et al., 2018). Many studies reported that fungi from the genus *Trichoderma* reduce oxidative stress in plants by

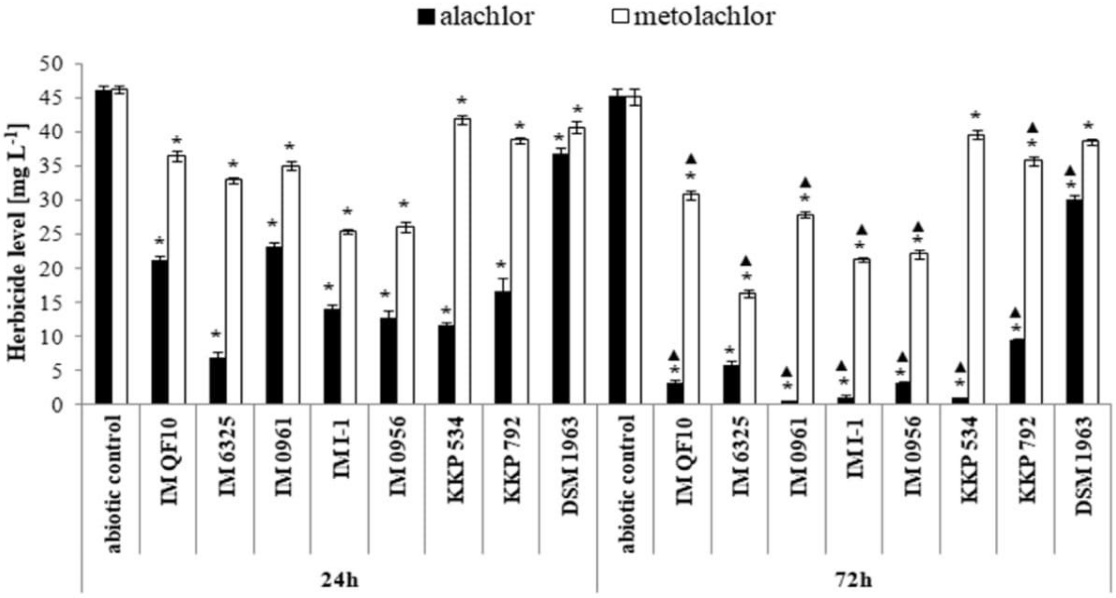

Fig. 2. Alachlor and metolachlor residues in *Trichoderma* spp. cultures after 24-and 72-h cultivation. Data are the mean ± SD ($n = 6$). * indicates the values that differ significantly from the biotic control at $P < 0.001$ and ▲ indicates the values that differ significantly in a time-dependent manner, according to the two-way ANOVA followed by Tukey's HSD test.

Table 1
Intracellular ROS generation ($O_2\cdot^-$ and HO·/$ONOO^-$) in *Trichoderma* spp. after 24 and 72-h exposure to chloroacetanilides. Data are the mean ± SD (n = 6). * indicates the values that differ significantly from the biotic control at $P < 0.001$ and ▲ indicates the values that differ significantly in a time-dependent manner, according to the two-way ANOVA followed by the Tukey's HSD test. BC - biotic control, A - alachlor, M - metolachlor.

Samples		HO·/$ONOO^-$ [%]		$O_2\cdot^-$ [%]	
		24 h	72 h	24 h	72 h
IM QF10	BC	0.03 ± 0.01	0.05 ± 0.02	0.64 ± 0.08	0.28 ± 0.08
	A	1.93 ± 0.42*	0.18 ± 0.06▲	3.50 ± 0.58*	0.31 ± 0.03▲
	M	0.56 ± 0.12	0.24 ± 0.01	2.66 ± 0.15	0.42 ± 0.17
IM I-1	BC	0.14 ± 0.01	0.05 ± 0.01	0.02 ± 0.01	0.00 ± 0.00
	A	0.44 ± 0.05	0.15 ± 0.21	0.32 ± 0.04	0.00 ± 0.00
	M	0.61 ± 0.07	0.82 ± 0.02	0.61 ± 0.02	0.29 ± 0.08
IM 0961	BC	0.06 ± 0.03	0.03 ± 0.01	0.59 ± 0.12	0.03 ± 0.01
	A	0.26 ± 0.02	0.19 ± 0.04	3.16 ± 0.32*	0.92 ± 0.04
	M	0.33 ± 0.01	0.52 ± 0.02	2.45 ± 0.23	1.82 ± 0.05
IM 0956	BC	0.31 ± 0.01	0.00 ± 0.00	3.65 ± 0.01	0.10 ± 0.00▲
	A	1.60 ± 0.02*	0.01 ± 0.00▲	29.54 ± 3.60*	0.19 ± 0.01▲
	M	0.47 ± 0.01	0.38 ± 0.08	11.83 ± 2.41*	0.73 ± 0.04▲
IM 6325	BC	0.14 ± 0.01	0.21 ± 0.02	5.35 ± 0.53	0.63 ± 0.07▲
	A	0.15 ± 0.01	0.19 ± 0.01	32.18 ± 0.97*	11.00 ± 0.72*▲
	M	0.65 ± 0.12	0.43 ± 0.01	10.63 ± 0.11*	10.60 ± 1.05*
KKP 534	BC	0.33 ± 0.06	0.03 ± 0.01	0.33 ± 0.02	0.05 ± 0.01
	A	0.88 ± 0.09	0.11 ± 0.02▲	5.19 ± 0.60*	0.26 ± 0.03▲
	M	2.83 ± 0.37*	0.33 ± 0.08▲	9.31 ± 0.10*	2.89 ± 0.16*▲
KKP 792	BC	0.21 ± 0.02	0.15 ± 0.01	0.38 ± 0.01	0.24 ± 0.04
	A	1.68 ± 0.25*	1.53 ± 0.30*	6.78 ± 0.92*	2.66 ± 0.51▲
	M	1.09 ± 0.11*	1.73 ± 0.49*	6.46 ± 0.46*	1.67 ± 0.50▲
DSM 1963	BC	0.55 ± 0.01	0.25 ± 0.01	0.60 ± 0.02	0.00 ± 0.00
	A	13.81 ± 1.61*	2.45 ± 0.28*▲	11.30 ± 0.68*	0.42 ± 0.01▲
	M	12.37 ± 0.40*	0.68 ± 0.05▲	13.78 ± 2.08*	1.32 ± 0.16▲

stimulation activity of ROS-scavenging enzymes (Gucel and Ahmad, 2015; Mastouri et al., 2010; Singh and Singh, 2011), but antioxidant response of *Trichoderma* fungi exposed to stress factors was unknown. Our results indicated that in most cases, the induced ROS formation occurred as a result of efficient biotransformation of the herbicides by the tested *Trichoderma* spp. Only in the IM I-1 cultures the presence of the herbicides had no effect on ROS production, although effective herbicide elimination was noted. Xu et al. (2008) suggested that ROS formation is caused by microbial metabolism in the case of biotransformation of another popular chloroacetanilide - acetochlor. Moreover, a study conducted by Słaba et al. (2015) showed that the overall ROS level in *P. marquandii* mycelia under 50 mg L^{-1} alachlor exposure did not increase until 24 h, reached the maximum at 48 h of the culture and then remained at a constant level. In turn, Rattanawong et al. (2015) revealed that 15 min exposure of *S. cerevisiae* to 2,4-dichlorophenoxyacetic acid (2,4-D) resulted in a high accumulation of intracellular ROS, whereas alachlor treatment of yeast did not increase the ROS level during the first 2 h of incubation. On the basis of these results authors suggested that alachlor has no pro-oxidant activity. Our results seem to confirm the presumption that ROS induction and intracellular accumulation are caused by fungal metabolism, active during chloroacetanilides conversion and that these herbicides have no direct pro-oxidant activity.

3.2. Lipids and protein oxidation caused by chloroacetanilides exposure

A common target for extensive oxidation, caused by the overproduction of ROS, are membrane lipids or endogenous proteins (Sies et al., 2017). To determine whether the chloroacetanilides exposure resulted in oxidative damage to proteins and lipids in *Trichoderma* spp. mycelia, we evaluated the content of the carbonyls groups and TBARS, respectively (Fig. 3). The effect of alachlor and metolachlor on TBARS formation—popular biomarkers of lipid peroxidation in the tested fungi is shown in Fig. 3A. The two-way ANOVA results demonstrated that the interaction between chloroacetanilides treatment and incubation time significantly affected the TBARS content in tested *Trichoderma* spp. (F = 86.52, $P < 0.001$). A noticeable, approximately 1.5– to 3-fold, enhancement in TBARS level in herbicides-treated fungal mycelia was observed throughout the culture period and confirmed the induction of oxidative stress. On the other hand, no significant variation in oxidative damage to lipids was noted in DSM 1963 exposed to both tested herbicides. Our results suggest that increased lipid peroxidation in tested *Trichoderma* strains was caused by the enhanced generation of reactive oxygen intermediates at the first 24 h (Table 1). According to Li et al. (2009), the enhanced amount of TBARS in the samples from the stationary phase of growth may result from chain reactions involved in this process. Moreover, the increased lipid peroxidation in fungal mycelia from the stationary phase of growth was also observed in *Umbelopsis isabellina* exposed to a common herbicide, 2,4-dichlorophenoxyacetic acid (Bernat et al., 2018). As shown in Fig. 3A, a significant decrease in the TBARS content in a time-dependent manner was observed in alachlor-treated cultures of IM 0956 and KKP 792, and it was correlated with the lower level of ROS (Table 1). This observation indicated a neutralization of oxidative damage by antioxidant defense or adaptation of the above-mentioned strains to herbicide-stress factor.

The oxidative modification of proteins based on carbonyl group formation under tested chloroacetanilides exposure in *Trichoderma* strains is presented in Fig. 3B. The interaction between the incubation time and the exposure to the herbicides had a significant influence on protein carbonyl content by *Trichoderma* spp. (F = 29.04, $P < 0.001$), which was demonstrated in the two-way ANOVA test. The level of protein carbonyl content in the control cultures of the tested fungal strains did not change during the experimental period. An increase in the protein oxidation was detected in the alachlor-treated cultures of IM 6325, IM I-1, and KKP 792 and the metolachlor-treated cultures of IM QF10, IM 6325, IM I-1, IM 0956, and DSM 1963 from the exponential phase of growth. However, for IM 0961 and KKP 534 with the addition of the herbicides, there were no significant changes in the carbonyl group content during the culture period. The accelerated protein oxidation at the first day of incubation was also correlated with overproduction of ROS (Table 1) and was a further evidence that confirmed chloroacetanilides-induced oxidative stress in *Trichoderma* strains. The addition of paraquat-enhanced protein carbonylation in the fungal strains *Humicola lutea* (Krumova et al., 2009) and *Aspergillus niger*

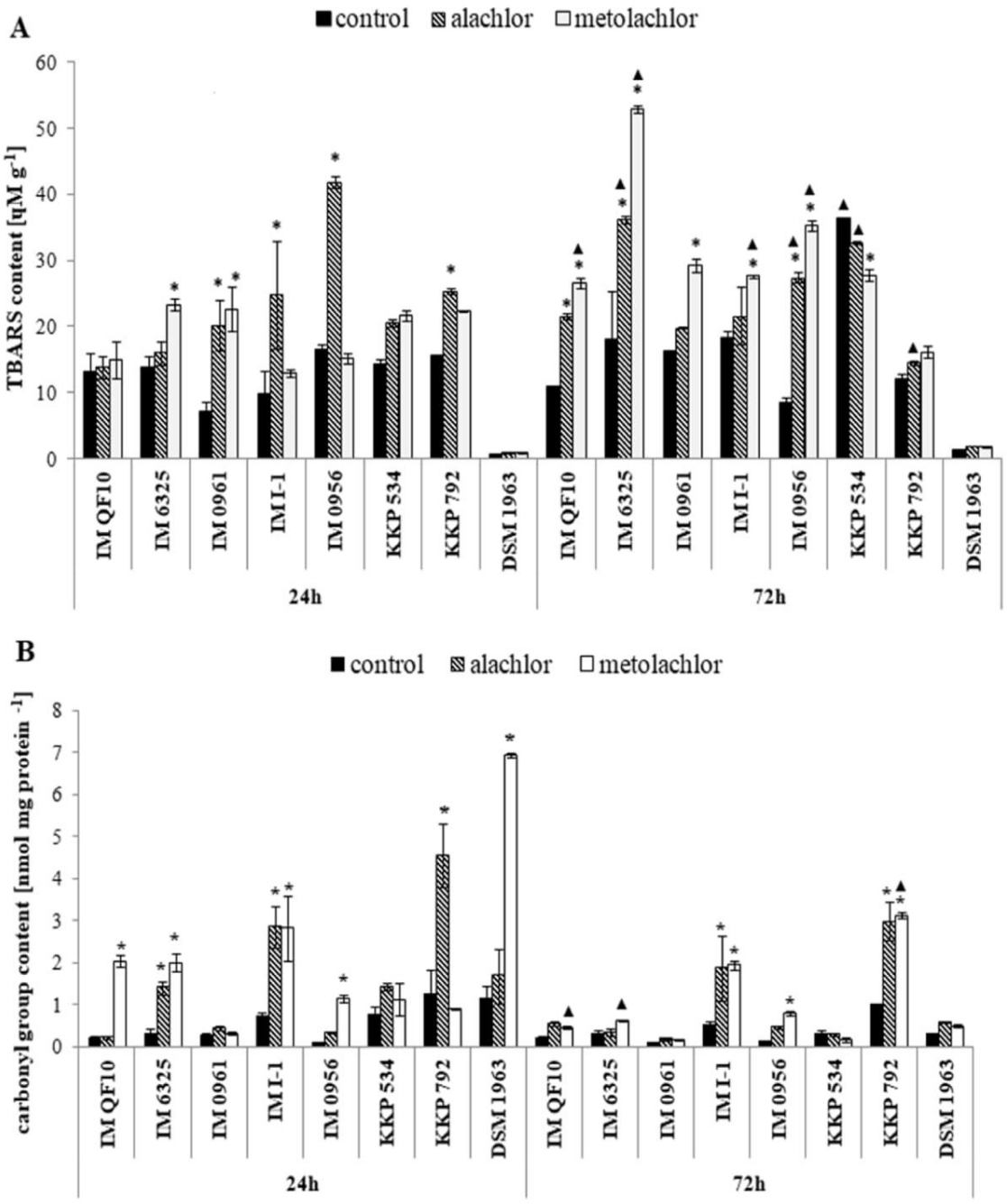

Fig. 3. The TBARS (A) and carbonyl group (B) content in *Trichoderma* spp. biomass exposed to chloroacetanilides. Data are the mean ± SD ($n = 6$). * indicates the values that differ significantly from the biotic control at $P < 0.001$ and ▲ indicates the values that differ significantly in a time-dependent manner, according to the two-way ANOVA followed by Tukey's HSD test.

(Angelova et al., 2005). However, after 72 h, the accumulation of the carbonyl group significantly decreased in the above-mentioned fungal cultures, which indicate an active response from the antioxidant system of the tested strains. Moreover, it has been suggested that oxidatively modified proteins may be effectively removed through intracellular proteolytic elimination, and proteases have been proposed as "secondary antioxidant defenses" in microorganisms (Nyström, 2005). Li et al. (2008) reported the enhanced proteolytic activity in oxygen-enrichment batch cultures of *A. niger* BD-1 in response to the rise in the level of carbonylated proteins.

3.3. Influence of chloroacetanilides on the phospholipids profile and membrane conditions in fungal cultures

The mode of action of chloroacetanilide herbicides, including alachlor and metolachlor, is the inhibition of the biosynthesis of lipids and fatty acids, especially very long fatty-acid chains (VLCFAs), which in turn affects cell membrane integrity (Matthes and Böger, 2002). The alterations in membrane lipid composition, especially phospholipids play an important role in the adaptation to different stress conditions (Bernat et al., 2018; Kroon et al., 2013; Słaba et al., 2013a, 2013b; Stolarek et al., 2019; Zawadzka et al., 2017). Therefore, the effect of the tested chloroacetanilides on the PL profile in *Trichoderma* spp. was observed (Fig. 4). Using the chromatographic technique, 34 species of tested fungi phospholipids from five classes was identified. Phosphatidylcholines (PC) were prevalent and constituted 31.63–82.23% of the total PLs content. Phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol, and phosphatidylserine (PS) were less abundant PLs, representing approximately 7.12–37.42, 0.41–22.85, 0.71–12.48, and 0.31–6.59%, respectively. The three-way ANOVA revealed that the interaction between the herbicide exposure, the growth phase, and the content of PLs species had a significant influence on the

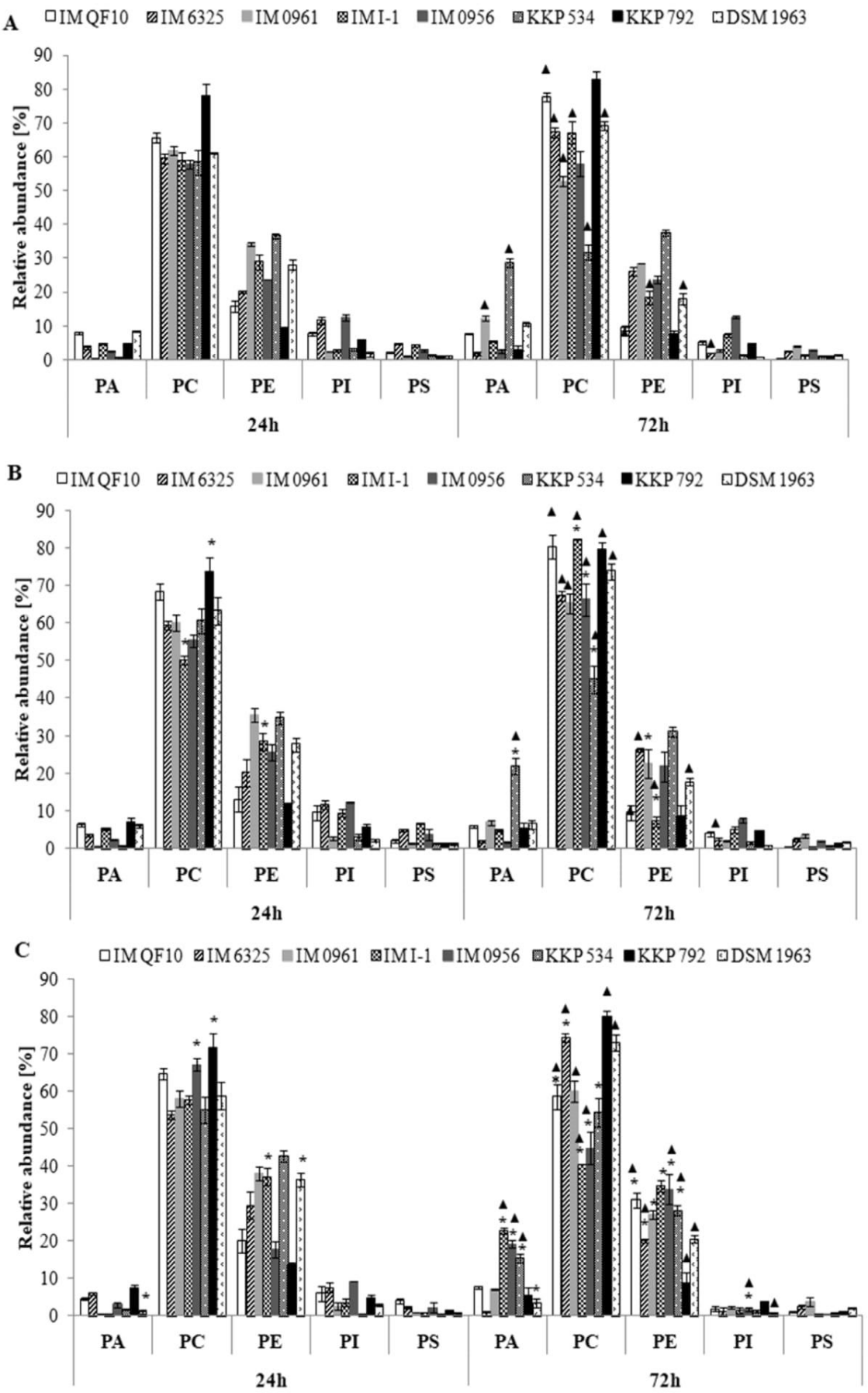

Fig. 4. The phospholipids composition of the tested *Trichoderma* spp. exposed to the chloroacetanilide herbicides (A—biotic controls; B—alachlor; C—metolachlor). PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine. Data are the mean ± SD ($n = 6$). * indicates the values that differ significantly from the biotic control at $P < 0.001$ and ▲ indicates the values that differ significantly in a time-dependent manner, according to the two-way ANOVA followed by Tukey's HSD test.

Trichoderma strains' phospholipids profile ($F = 87.03$, $P < 0.001$ for alachlor and $F = 69.70$, $P < 0.001$ for metolachlor). In the exponential phase of growth (24 h) fungal lipids composition after alachlor or metolachlor exposure was comparable to the control samples. A significant change in the phospholipids content in *Trichoderma* mycelia was observed after 72 h of incubation. In alachlor-treated cultures of IM 0961, IM I-1, IM 0956, and KKP 534, 14.46%, 15.15%, 8.36%, and 13.28% increase in the PC level related to 5.77, 11.13, 1.89 and 6.33% decrease in the PE level was observed, respectively. The same correlation between the phosphatidylcholine and phosphatidylethanolamine content was observed in IM 6325, IM 0961, and KKP 534 supplemented with metolachlor. A reverse dependence was noted in the cultures of IM QF10, IM I-1, and IM 0956 with the addition of metolachlor, a 12.96–26.59% decrease in the PC content and 6.10–16.45% increase in PE content was noted. Phosphatidylcholine and phosphatidylethanolamine are nearly related, because PC can be synthesized from PE (Xia et al., 2011). According to Li et al. (2006), the proportion between PC and PE may provide information about membrane integrity. An increase in PC level stabilizes the lipid bilayer, while PE has the tendency to form hexagonal phase, thus reducing the membrane fluidity. Therefore, the differences in the PC/PE ratio after incubation of the *Trichoderma* spp. with chloroacetanilides were determined (Table 2). The pronounced changes in this index were noted in the stationary phase of growth. Alachlor caused an increase in the PC/PE ratio in IM 0961 (from 1.86 to 2.88), IM I-1 (from 3.68 to 11.55), IM 0956 (from 2.44 to 3.04), and KKP 534 (from 0.85 to 1.45) mycelia. Moreover, a higher value of the PC/PE ratio in metolachlor-treated cultures of IM 6325 and KKP 534 was also noted. These results point to an increase in the membrane fluidity of the above-mentioned fungi. Such phenomenon may lead to enhancement of available herbicides for the fungal cells, which can be important in the effective biotransformation of these compounds. In the case of another filamentous fungus *Cunninghamella elegans*, an increase in the membrane fluidity was noted under tributyltin (TBT) exposure (Bernat et al., 2014). Moreover, metolachlor in the cultures of IM QF10, IM I-1, and IM 0956 caused approximately 2–4-fold decrease in the PC/PE ratio. It has been suggested that membrane integrity loss results from the decrease in the proportion between PC and PE (Li et al., 2006). Nevertheless, a reduction in membrane fluidity in above-mentioned *Trichoderma* strains may evidence an adaptive mechanism that enables a gradual uptake of metolachlor. Stolarek et al. (2019) also reported a decline in the value of PC/PE ratio in *Metarhizium robertsii* under dibutyltin (DBT) and TBT exposure. In the present study, the addition of metolachlor to IM I-1 and IM 0956 cultures caused a significant increase in PA content. Phosphatidic acid is known as signal lipid, and in response to different abiotic and biotic stress factors, PA level can significantly be enhanced (Testerink and Munnik, 2005). An increased amount of PA in carbazole-treated *C. elegans* mycelia (Zawadzka et al., 2015) and DBT- or TBT-treated *M. robertsii* (Stolarek et al., 2019) cells was recently reported. The changes in the content of most abundant PLs species and PC/PE ratio suggested a significant effect of tested chloroacetanilides on the membrane composition. Among the tested chloroacetanilides, metolachlor clearly modified the PLs in *Trichoderma* spp. in the stationary phase of growth, which was manifested through the difference in PA, PE and PC levels. The results point to a defense mechanism of tested microorganisms to chloroacetanilide-induced stress, which may also be related to high tolerance of *Trichoderma* spp. to this toxic compound.

Table 2
Effect of the chloroacetanilide herbicides on membrane permeability and the PC/PE ratio in *Trichoderma* spp. after 24- and 72-h exposure to chloroacetanilides. Data are the mean ± SD ($n = 6$). * indicates the values that differ significantly from the biotic control at $P < 0.001$ and ▲ indicates the values that differ significantly in a time-dependent manner, according to the two-way ANOVA followed by the Tukey's HSD test. BC - biotic control, A - alachlor, M - metolachlor.

Samples		Membrane permeability [%]		PC/PE ratio	
		24 h	72 h	24 h	72 h
IM QF10	BC	0.26 ± 0.04	0.05 ± 0.01	4.19	8.27
	A	0.64 ± 0.11	0.43 ± 0.03	5.22	8.43
	M	0.81 ± 0.11	0.54 ± 0.08	3.24	1.89
IM I-1	BC	0.09 ± 0.01	0.21 ± 0.02	2.03	3.68
	A	0.66 ± 0.07	0.63 ± 0.07	1.76	11.55
	M	0.88 ± 0.12*	1.42 ± 0.03*	1.55	1.17
IM 0961	BC	0.57 ± 0.05	0.59 ± 0.21	1.82	1.86
	A	1.60 ± 0.20*	0.17 ± 0.05▲	1.69	2.88
	M	2.49 ± 0.34*	1.29 ± 0.12▲	1.53	2.23
IM 0956	BC	1.91 ± 0.04	0.22 ± 0.01▲	2.44	2.44
	A	2.22 ± 0.20	1.56 ± 0.35*	2.16	3.04
	M	3.02 ± 0.69*	2.62 ± 0.86*	3.81	1.33
IM 6325	BC	0.70 ± 0.15	0.09 ± 0.01	2.96	2.58
	A	1.70 ± 0.23*	1.01 ± 0.08*	2.96	2.58
	M	3.10 ± 0.35*	0.64 ± 0.05▲	1.83	3.72
KKP 534	BC	1.86 ± 0.09	0.40 ± 0.02▲	1.60	0.85
	A	1.91 ± 0.27	0.71 ± 0.14▲	1.74	1.45
	M	1.17 ± 0.22	1.31 ± 0.16*	1.29	1.93
KKP 792	BC	0.38 ± 0.04	0.99 ± 0.37	8.65	10.73
	A	0.88 ± 0.01	1.27 ± 0.29	6.36	9.28
	M	0.72 ± 0.02	1.47 ± 0.25▲	5.22	9.20
DSM 1963	BC	0.36 ± 0.05	0.94 ± 0.18	2.17	3.84
	A	1.04 ± 0.30*	1.69 ± 0.12	2.29	4.19
	M	0.64 ± 0.01	3.19 ± 0.09*▲	1.62	3.57

The condition of the fungal membrane exposed to alachlor or metolachlor was determined by the measurement of propidium iodide intracellular accumulation on *Trichoderma* mycelium from the exponential and stationary growth phases (Table 2). The presence of the red dye inside the fungal hyphae indicated disruptions in membrane integrity. The two-way ANOVA showed that the interaction between the herbicides exposure and the growth phase ($F = 25.12$, $P < 0.001$) had a significant influence on the permeability of the tested *Trichoderma* spp. membrane. As illustrated in Table 2, alachlor and metolachlor caused an increase in the membrane permeability of the tested fungi throughout the culture period, but in most cases without statistical significance. Despite the overproduction of TBARS and changes in the phospholipids composition under chloroacetanilides exposure in the stationary phase of growth, we did not observe a strong disruption of *Trichoderma* spp. membrane integrity.

3.4. The role of antioxidant enzymes in response to chloroacetanilides-mediated oxidative stress

Catalase (CAT) and superoxide dismutase (SOD) belong to the group of antioxidant enzymes with confirmed significance in the alleviation of the reactive molecules level, and the activity is frequently evidence for oxidative stress response (Sies et al., 2017) (). Therefore, the CAT and SOD activities in the mycelia of *Trichoderma* spp. under alachlor or metolachlor exposure were examined (Fig. 5). The interaction between the chloroacetanilides treatment and the phase of growth had a significant influence on CAT and SOD activity in fungal cultures ($F = 129.40$, $P < 0.001$ and $F = 18.96$, $P < 0.001$, respectively), which was demonstrated in the two-way ANOVA test. The analyzed antioxidant enzymes of tested *Trichoderma* strains reacted differently under chloroacetanilides-induced oxidative stress depending on the incubation time. No alterations were observed in the catalase level in IM 6325, KKP 534, KKP 792, and DSM 1963 exposed to alachlor and metolachlor in the exponential phase growth (Fig. 5A). However, after 72 h of exposure to the herbicides 2–6-fold increase in the CAT activity was observed in the IM QF10, IM 6325, IM 0961, KKP 534, KKP 792, and DSM 1963 cultures compared to the samples from 24 h of incubation. These results were related to the alleviated levels of the reactive molecules in the tested fungal mycelia from the stationary phase (Table 1) and indicate an active response from the antioxidant system of the tested fungi. In contrast, despite significant increase in the content of superoxide anion (Table 1) no major changes in the SOD activity

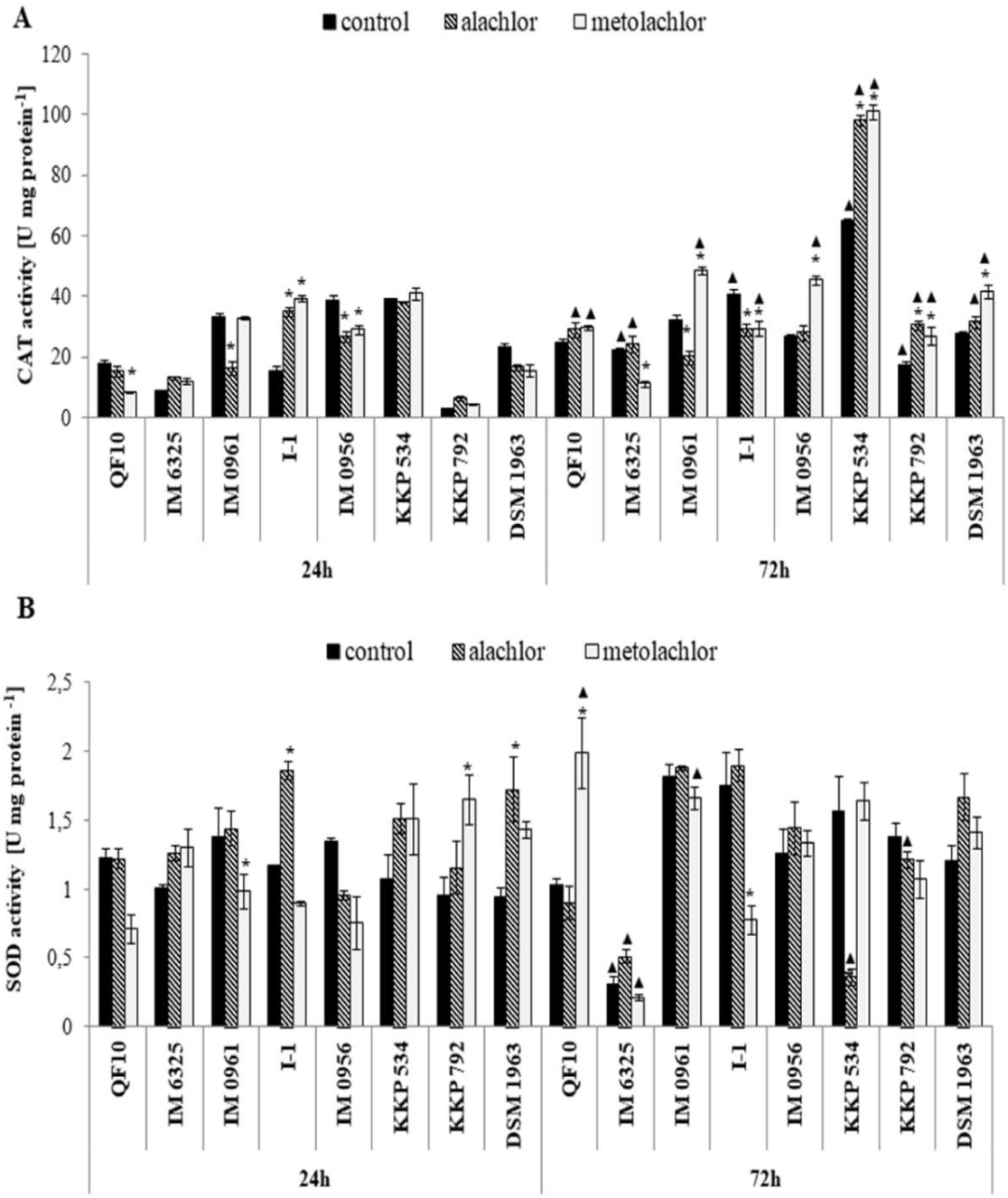

Fig. 5. Catalase (A) and superoxide dismutase (B) activity in *Trichoderma* spp. under chloroacetanilides exposure. Data are the mean ± SD ($n = 6$). * indicates the values that differ significantly from the biotic control at $P < 0.001$ and ▲ indicates the values that differ significantly in a time-dependent manner, according to the two-way ANOVA followed by the Tukey's HSD test.

in the most fungal cultures are exposed to alachlor or metolachlor were observed (Fig. 5B). Only in alachlor-treated cultures of IM I-1 and DSM 1963 and in metolachlor-treated cultures of KKP 792, stimulation for the SOD activity after 24 h of incubation was noted and related to the elimination of reactive molecules from the fungal cells (Table 1). Probably, in the other *Trichoderma* strains, the applied concentration of tested chloroacetanilides was insufficient to observe a significant increase in dismutase activity. However, the antioxidant system of tested *Trichoderma* strains could still effectively remove $O_2^{\cdot -}$ (Table 1). For the alachlor-degrading fungus *P. marquandii,* the CAT and SOD activities significantly decreased in the mycelia from exponential phase of mycelia growth, but after this period was comparable to the control (Słaba et al., 2015). Literature data indicated that some *Trichoderma* spp. were found to be effective in the mitigation of pathogen- or pesticide-induced oxidative stress in agricultural crops, e.g., tomato (Mastouri et al., 2012), sunflower (Singh and Singh, 2011), or wheat (Mohapatra and Mittra, 2017) by the activation of antioxidant enzymes such as catalase, superoxide dismutase or peroxide ascorbate. However, to the best to our knowledge, there is a lack of information about the antioxidant response of these microorganisms to environmental stress factors.

4. Conclusion

In summary, the overproduction of intracellular reactive oxygen species (especially the superoxide anion) and increased lipid and protein oxidative damage confirmed chloroacetanilide-induced stress in *Trichoderma* spp. cultures. Our results also indicated that induced ROS formation occurred as a result of the metabolism of the herbicides. However, the mitigation in the content of intracellular ROS during the

biotransformation of alachlor and metolachlor suggests an active antioxidant response and also the mechanism of tolerance in the tested fungi. Moreover, the decline in the content of protein oxidative damage biomarker was alleviated in the stationary *Trichoderma* spp. cultures. Catalase activity was involved in active fungal defense against induced oxidative stress caused by chloroacetanilides. This study documented for the first time the effect of alachlor and metolachlor on the PLs composition and membrane integrity in non-target organisms such as filamentous fungi. The changes in the overall content of two main PLs group (PC and PE) can be considered as defense response of fungal cells against the toxic action of chloroacetanilides and also may be connected with a high tolerance of *Trichoderma* spp. to the herbicides. These findings may contribute to broadening the knowledge about the mechanisms of cellular response to oxidative stress in chloroacetanilide-degrading microorganisms.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.109629.

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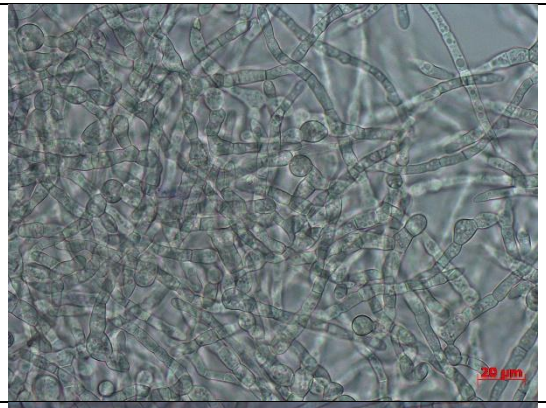
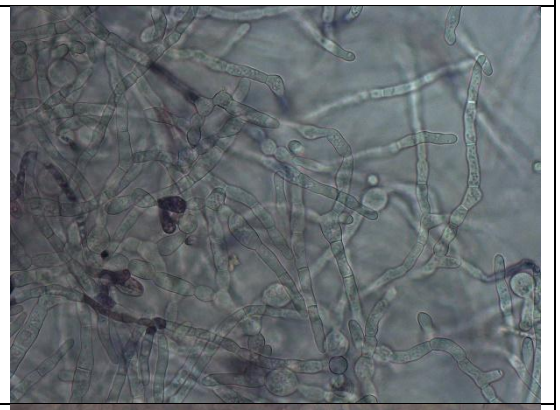
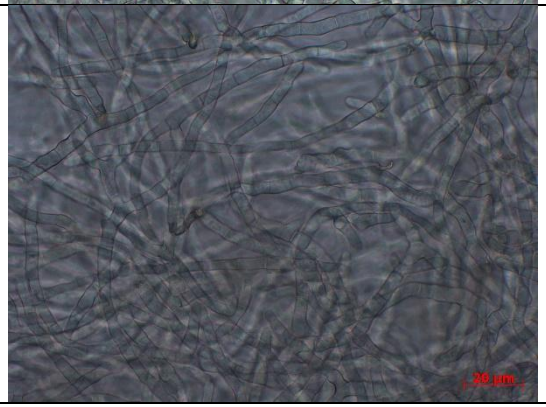
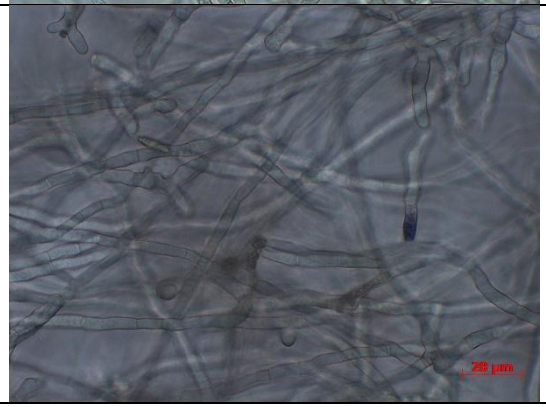
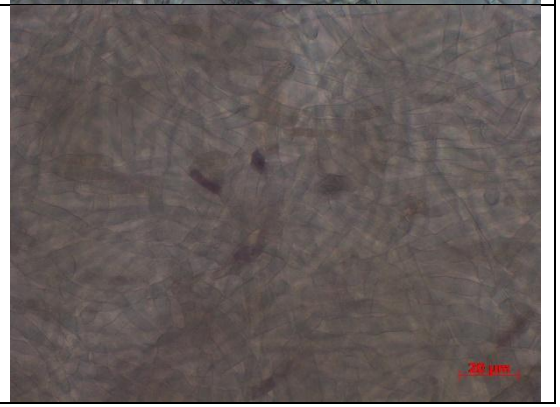
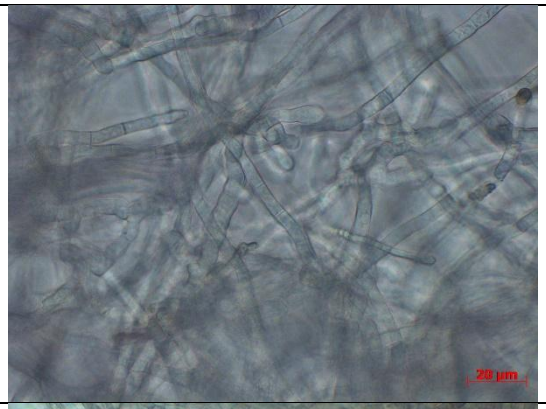
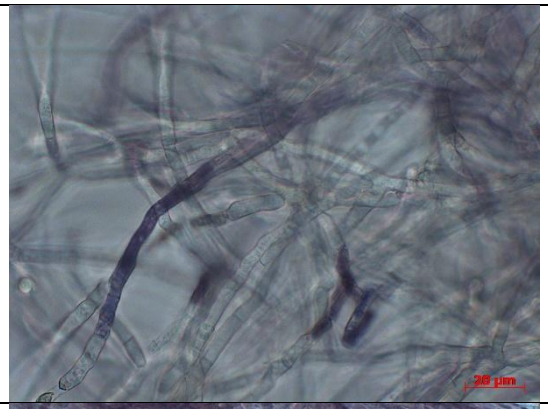
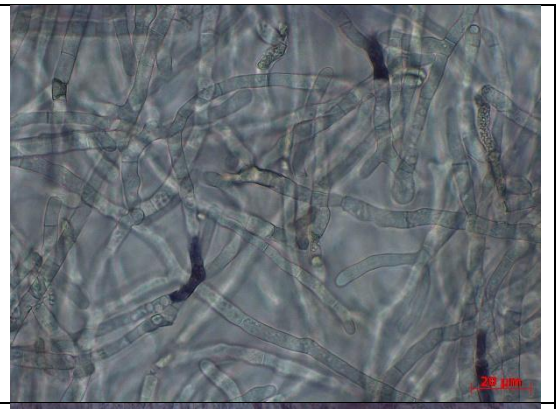
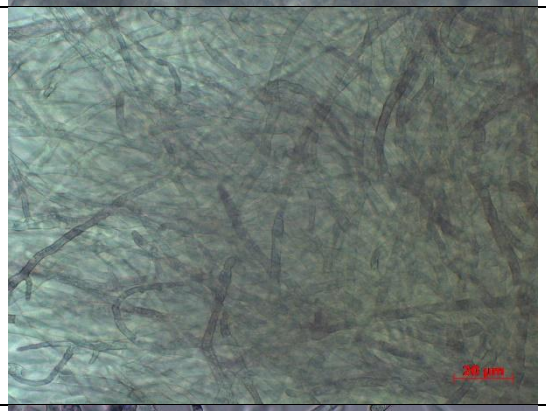
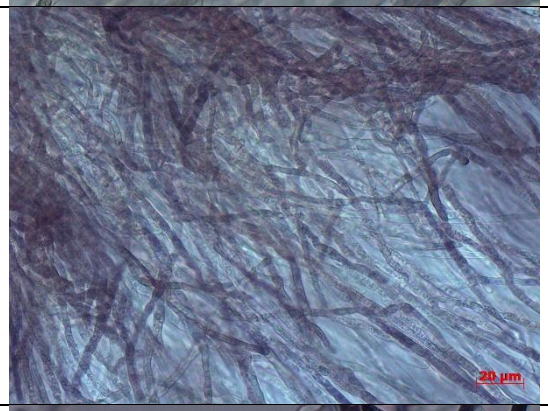
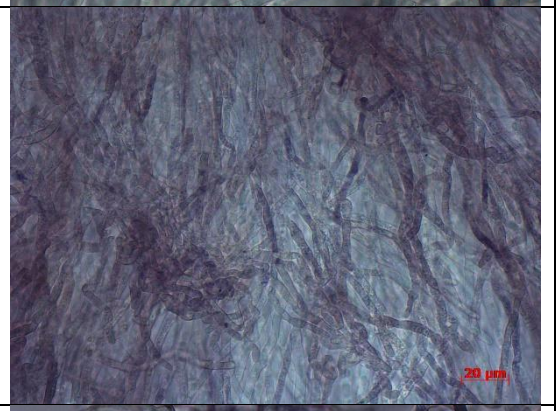
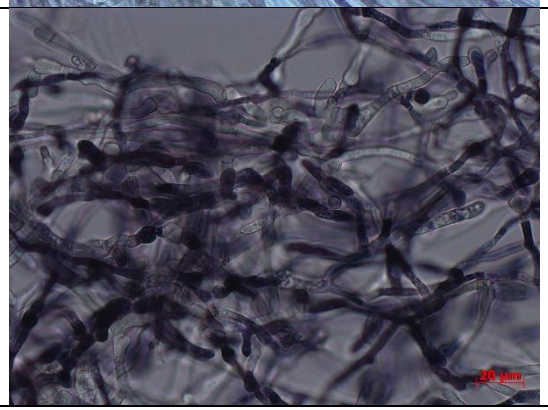
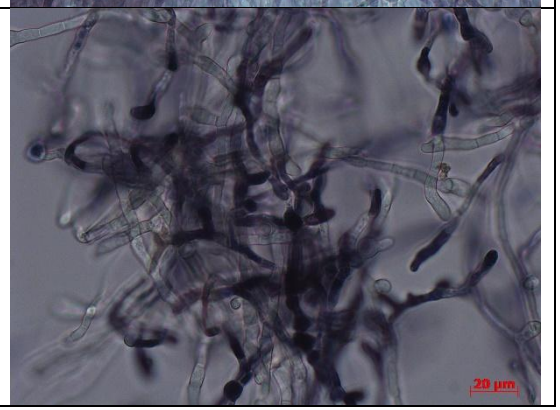
Supplementary

Table S1 Intracellular superoxide anion ($O_2^{\bullet-}$) generation in *Trichoderma* spp. after 24-h exposure to chloroacetanilides. Bar represents 20 µm.

	Biotic control	Alachlor	Metolachlor
IM QF10			
IM I-1			

IM 0961			
IM0956			
IM 6325			

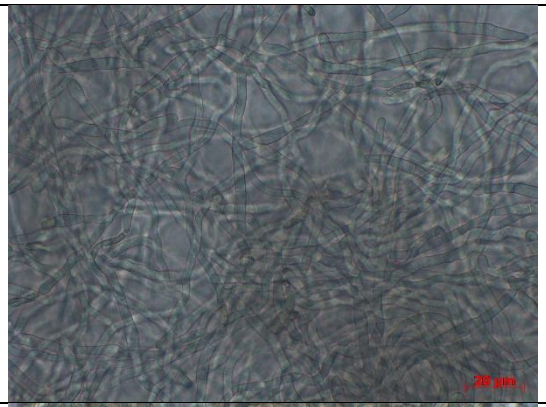
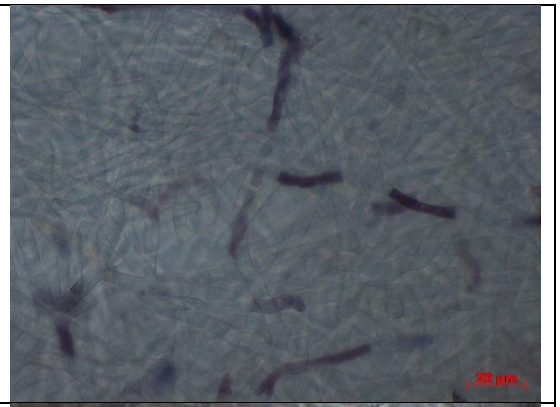
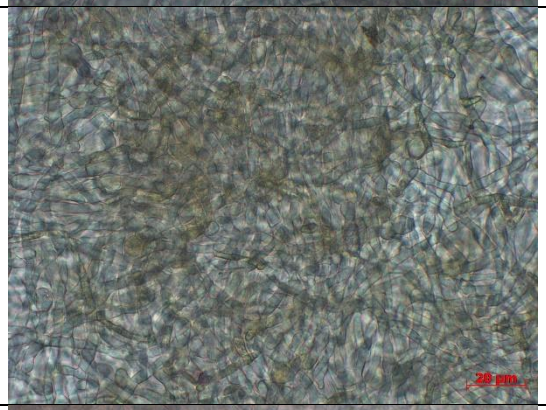
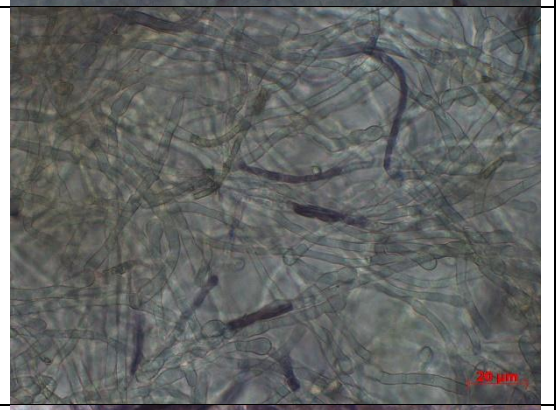
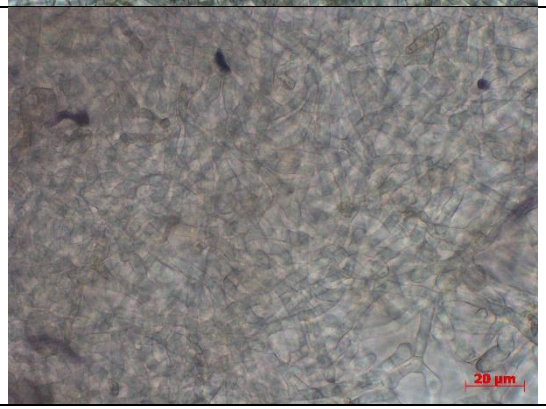
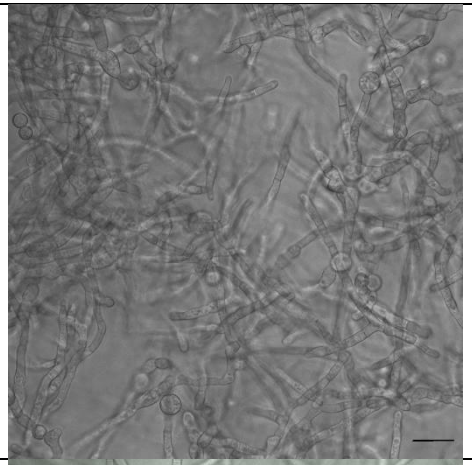
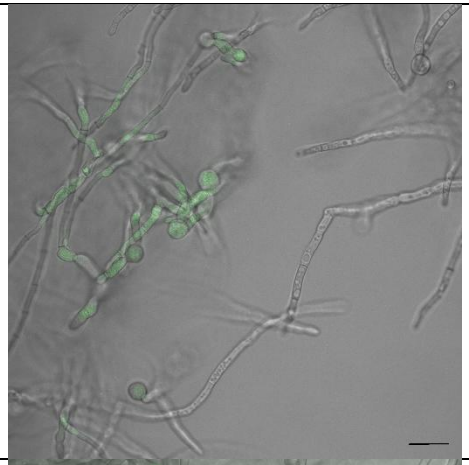
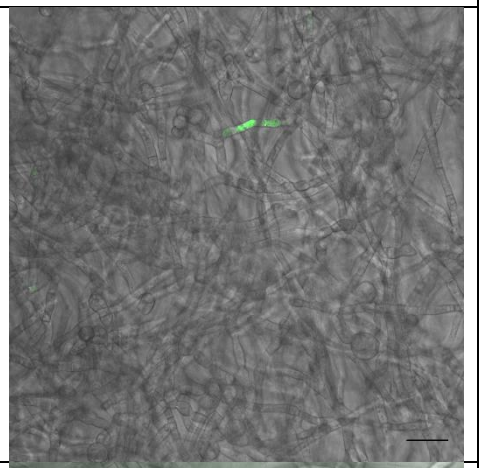
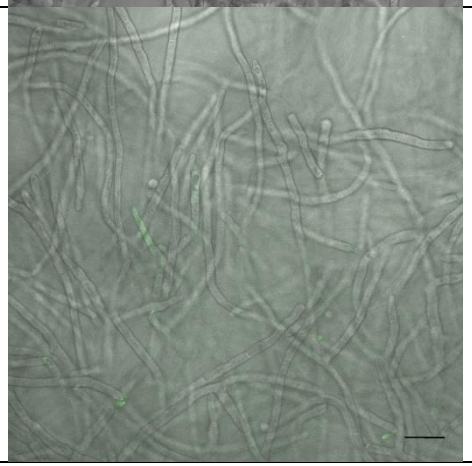
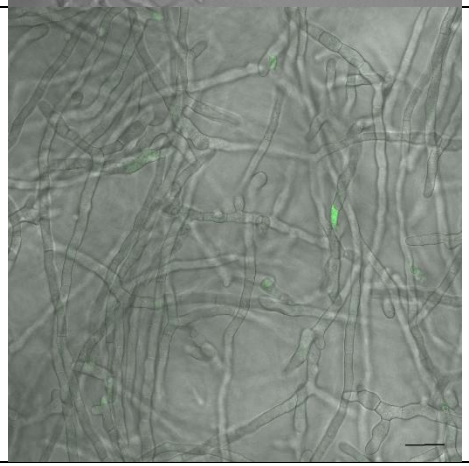
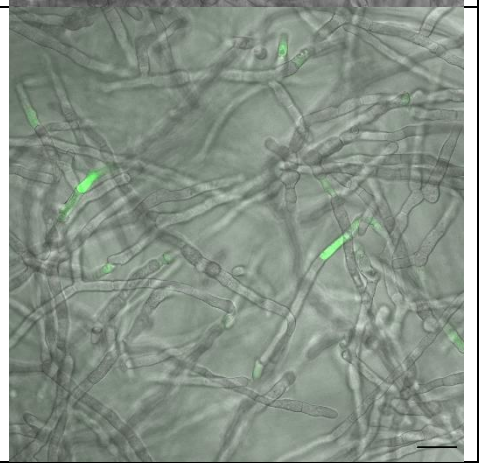
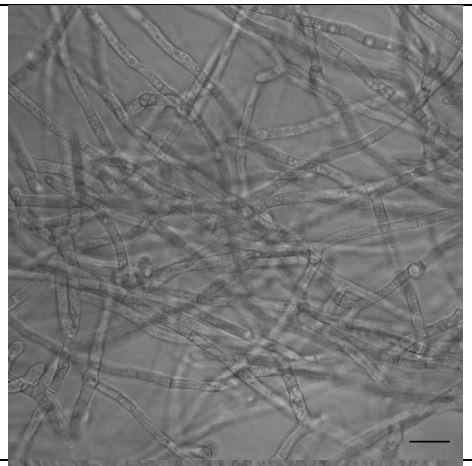
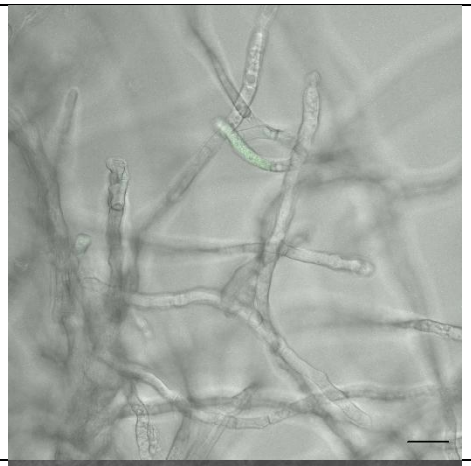
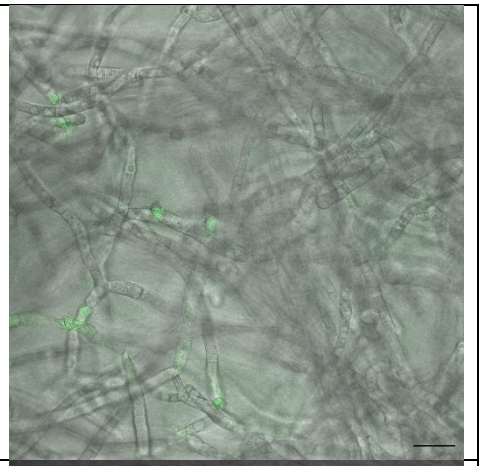
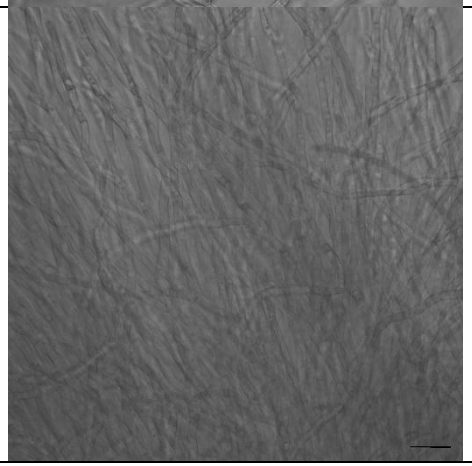
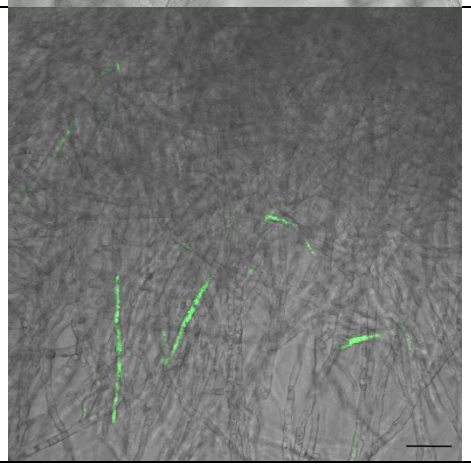
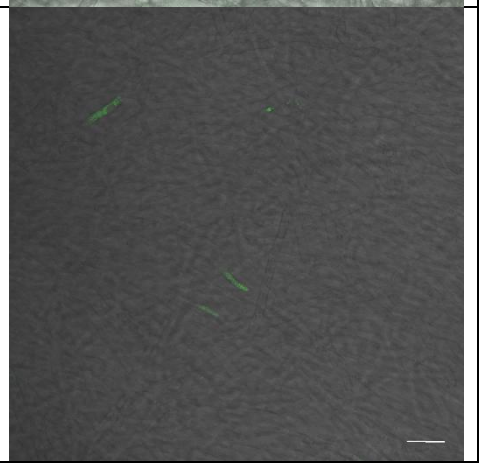
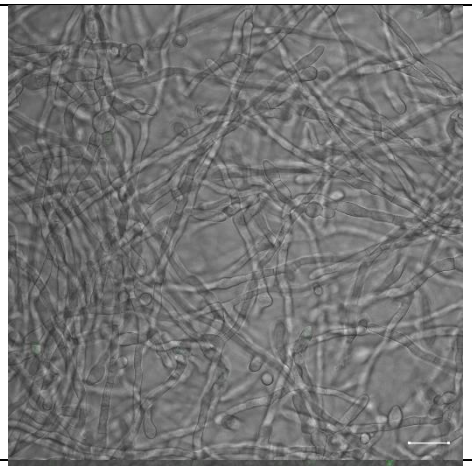
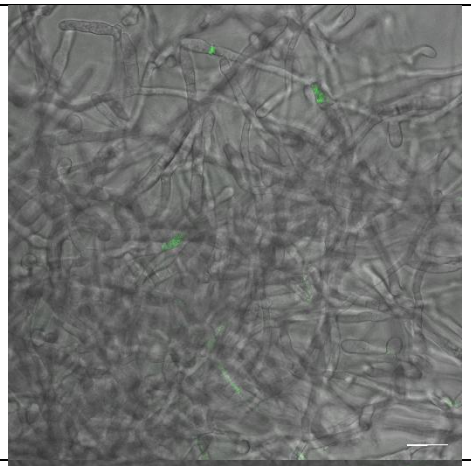
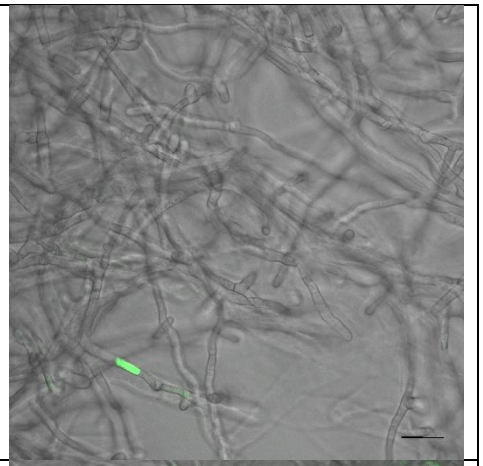
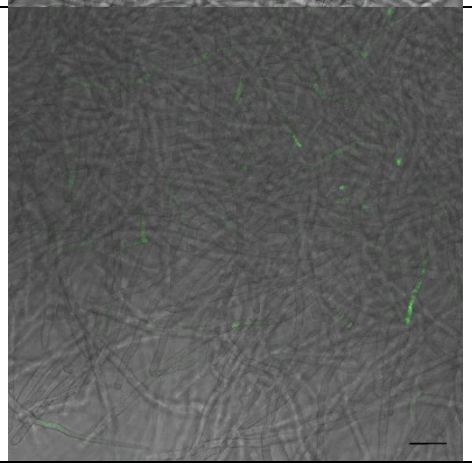
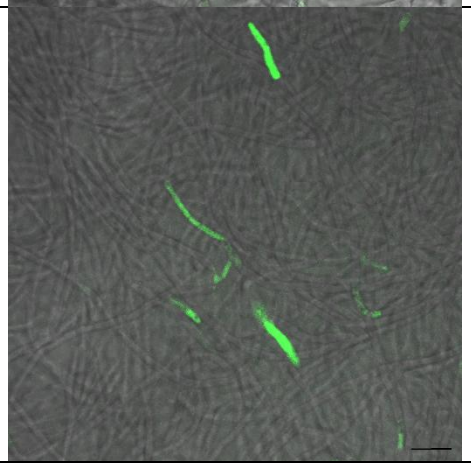
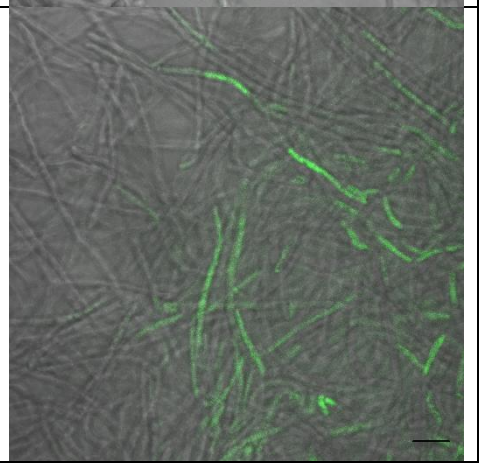
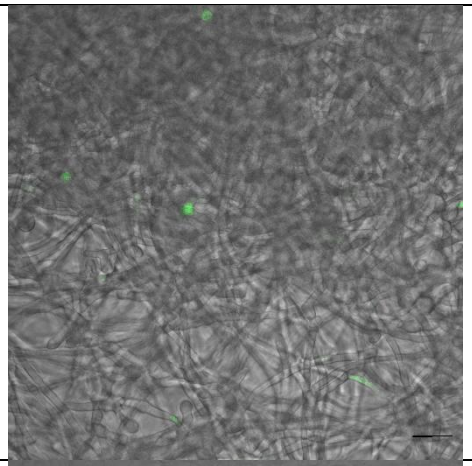
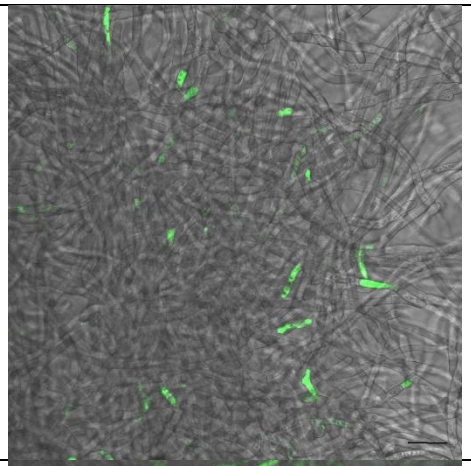
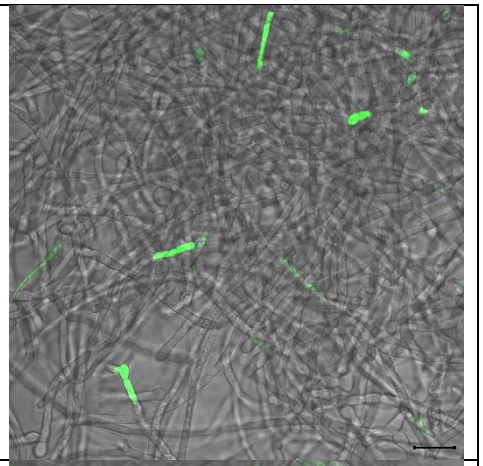
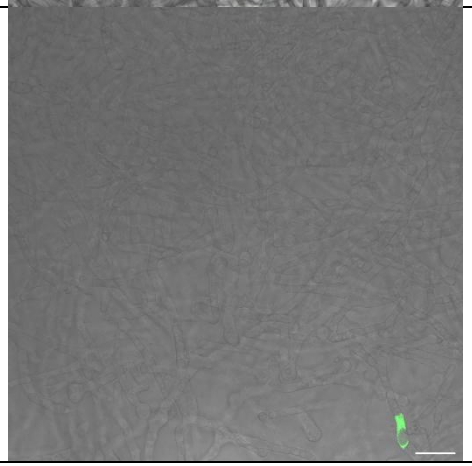
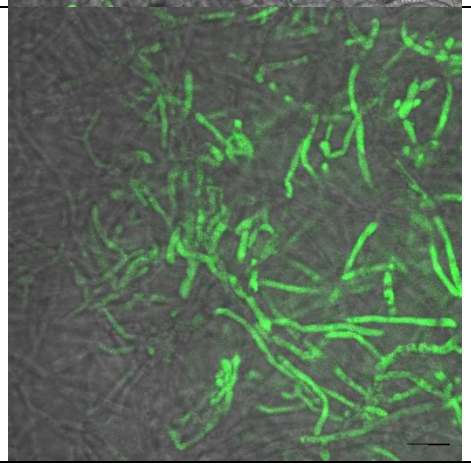
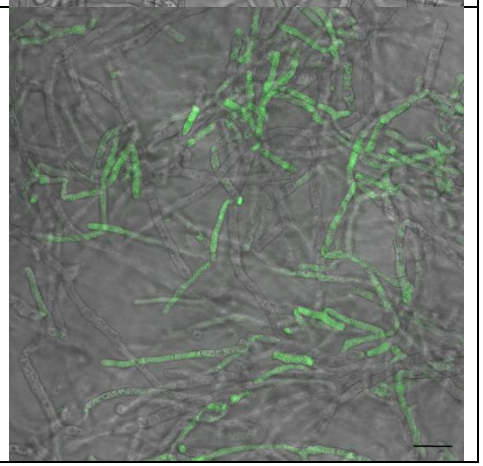
KKP 534			
KKP 792			
DSM 1963			

Table S2 Intracellular peroxynitrate anion/hydroxyl radical ($HO^{\bullet}/ONOO^{-}$**)** generation in *Trichoderma* spp. after 24-h exposure to chloroacetanilides. Bar represents 20 µm.

	Biotic control	**Alachlor**	**Metolachlor**
IM QF10			
IM I-1			

IM 0961			
IM0956			

IM 6325			
KKP 534			

KKP 792			
DSM 1963			

Oświadczenia współautorów

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Katedra Mikrobiologii
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Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

OŚWIADCZENIE

Oświadczam, że w pracy:

Nykiel-Szymańska J., Bernat P., Słaba M. (w druku). Biotransformation and detoxification of chloroacetanilide herbicides by *Trichoderma* spp. with plant growth-promoting activities. Pesticide Biochemistry and Physiology, 10.1016/j.pestbp.2019.11.018.

Mój wkład wynosił 80% i dotyczył współudziału w opracowaniu koncepcji badań, zaplanowaniu eksperymentów, wykonaniu wszystkich eksperymentów przedstawionych w pracy, tj. ilościowa i jakościowa analiza rozkładu alachloru i metolachloru przez osiem szczepów *Trichoderma*, ocena toksyczności produktów mikrobiologicznej degradacji herbicydów, analiza zdolności szczepów *Trichoderma* w promowaniu wzrostu siewek rzepaku poddanych ekspozycji na alachlor i metolachlor; opracowaniu i interpretacji uzyskanych wyników, analizie statystycznej przedstawionych wyników oraz współudziale w pisaniu manuskryptu. Ponadto byłam kierownikiem projektu, z którego były finansowane badania opisane w niniejszym manuskrypcie.

Justyna Nykiel-Szymańska

.....

Podpis

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Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

OŚWIADCZENIE

Oświadczam, że w pracy:

Nykiel-Szymańska J., Bernat P., Słaba M. (w druku). Biotransformation and detoxification of chloroacetanilide herbicides by *Trichoderma* spp. with plant growth-promoting activities. Pesticide Biochemistry and Physiology, 10.1016/j.pestbp.2019.11.018.

Mój wkład wynosił 10% i dotyczył współudziału w wykonywaniu eksperymentów dotyczących ilościowej i jakościowej analizy biotransformacji herbicydów chloroacetanilidowych oraz korekcie manuskryptu.

.....

Podpis

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Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

OŚWIADCZENIE

Oświadczam, że w pracy:

Nykiel-Szymańska J., Bernat P., Słaba M. (w druku) Biotransformation and detoxification of chloroacetanilide herbicides by *Trichoderma* spp. with plant growth-promoting activities. Pesticide Biochemistry and Physiology, 10.1016/j.pestbp.2019.11.018.

Mój wkład wynosił 10% i dotyczył współudziału w opracowaniu koncepcji badań, planowaniu eksperymentów, konsultacjach naukowych oraz korekcie manuskryptu.

.....

Podpis

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OŚWIADCZENIE

Oświadczam, że w pracy:

Nykiel-Szymańska J., Bernat B., Słaba M. 2018. Potential of *Trichoderma koningii* to eliminate alachlor in the presence of copper ions. Ecotoxicology and Environmental Safety 162, 1-9.

Mój wkład wynosił 65% i dotyczył współudziału w zaplanowaniu koncepcji badań, przebiegu eksperymentów, wykonaniu wszystkich eksperymentów przedstawionych w pracy, tj. ilościowa i jakościowa analiza rozkładu alachloru przez mikroskopowy grzyb strzępkowy *T. koningii*, ocena toksyczności produktów mikrobiologicznej degradacji alachloru, aktywność lakazy w hodowlach *T. koningii* z dodatkiem alachloru oraz wpływ miedzi na eliminację herbicydu przez badany szczep; opracowaniu i interpretacji uzyskanych wyników, analizie statystycznej przedstawionych wyników oraz współudziale w pisaniu manuskryptu. Ponadto byłam kierownikiem projektu, z którego były finansowane badania opisane w niniejszym manuskrypcie.

Justyna Nykiel-Szymańska

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Podpis

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OŚWIADCZENIE

Oświadczam, że w pracy:

Nykiel-Szymańska J., Bernat B., Słaba M. 2018. Potential of *Trichoderma koningii* to eliminate alachlor in the presence of copper ions. Ecotoxicology and Environmental Safety 162, 1-9.

Mój wkład wynosił 15% i dotyczył współudziału w wykonywaniu eksperymentów dotyczących ilościowej i jakościowej analizy biotransformacji alachloru, konsultacjach naukowych oraz współredagowaniu manuskryptu.

.....

Podpis

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Wydział Biologii i Ochrony Środowiska
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OŚWIADCZENIE

Oświadczam, że w pracy:

Nykiel-Szymańska J., Bernat B., Słaba M. 2018. Potential of *Trichoderma koningii* to eliminate alachlor in the presence of copper ions. Ecotoxicology and Environmental Safety 162, 1-9.

Mój wkład wynosił 20% i dotyczył współudziału w opracowaniu koncepcji badań, planowaniu eksperymentów, konsultacjach naukowych oraz współredagowaniu manuskryptu.

.....

Podpis

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OŚWIADCZENIE

Oświadczam, że w pracy:

Nykiel-Szymańska J., Różalska S., Bernat P., Słaba M. 2019. Assessment of oxidative stress and phospholipids alterations in chloroacetanilides-degrading *Trichoderma* spp.. Ecotoxicology and Environmental Safety 184, 10.1016/j.ecoenv.2019.109629.

Mój wkład wynosił 60% i dotyczy współudziału w zaplanowaniu koncepcji badań i przebiegu eksperymentów, wykonaniu eksperymentów przedstawionych w pracy, tj. detekcji reaktywnych form tlenu w hodowlach szczepów *Trichoderma* z dodatkiem alachloru i metolachloru, pomiar zawartości produktów peroksydacji lipidów oraz białek w hodowlach szczepów *Trichoderma* poddanych ekspozycji na badane herbicydy, pomiar aktywności enzymów antyoksydacyjnych w hodowlach *Trichoderma* spp. z dodatkiem herbicydów, analiza profilu fosfolipidów badanych szczepów poddanych działaniu alachloru i metolachloru, ocena wpływu badanych herbicydów na przepuszczalność błony komórkowej *Trichoderma* spp.; opracowaniu i interpretacji uzyskanych wyników, analizie statystycznej przedstawionych wyników oraz współudziale w pisaniu manuskryptu. Ponadto byłam kierownikiem projektu, z którego były finansowane badania opisane w niniejszym manuskrypcie.

Justyna Nykiel-Szymańska
.....
Podpis

Dr hab. Sylwia Różalska, prof. UŁ
Katedra Mikrobiologii
Przemysłowej i Biotechnologii
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

OŚWIADCZENIE

Oświadczam, że w pracy:

Nykiel-Szymańska J., Różalska S., Bernat P., Słaba M. 2019. Assessment of oxidative stress and phospholipids alterations in chloroacetanilides-degrading *Trichoderma* spp.. Ecotoxicology and Environmental Safety 184, 10.1016/j.ecoenv.2019.109629.

Mój wkład wynosił 15% i dotyczył udziału w wykonaniu oraz opracowaniu wyników z eksperymentów dotyczących detekcji reaktywnych form tlenu oraz pomiaru przepuszczalności błony komórkowej w hodowlach szczepów *Trichoderma* z dodatkiem alachloru i metolachloru.

Sylwia Różalska

.....

Podpis

Dr hab. Przemysław Bernat, prof. UŁ
Katedra Mikrobiologii
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Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

OŚWIADCZENIE

Oświadczam, że w pracy:

Nykiel-Szymańska J., Różalska S., Bernat P., Słaba M. 2019. Assessment of oxidative stress and phospholipids alterations in chloroacetanilides-degrading *Trichoderma* spp.. Ecotoxicology and Environmental Safety 184, 10.1016/j.ecoenv.2019.109629.

Mój wkład wynosił 10% i dotyczył współudziału w wykonywaniu eksperymentów dotyczących analizy profilu fosfolipidowego szczepów *Trichoderma* poddanych ekspozycji na herbicydy chloroacetanilidowe oraz korekcie manuskryptu.

.....

Podpis

Dr hab. Mirosława Słaba, prof. UŁ
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Przemysłowej i Biotechnologii
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

OŚWIADCZENIE

Oświadczam, że w pracy:

Nykiel-Szymańska J., Różalska S., Bernat P., Słaba M. 2019. Assessment of oxidative stress and phospholipids alterations in chloroacetanilides-degrading *Trichoderma* spp.. Ecotoxicology and Environmental Safety 184, 10.1016/j.ecoenv.2019.109629.

Mój wynosił 15% i dotyczył współudziału w koncepcji badań, planowaniu eksperymentów, konsultacjach naukowych oraz współredagowaniu manuskryptu.

.....

Podpis