

Stacjonarne Studia Doktoranckie Mikrobiologii,
Biotechnologii i Biologii Eksperymentalnej

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**Badanie aktywności
zewnątrzkomórkowych metabolitów
Trichoderma harzianum jako
naturalnych elicytorów, z użyciem
technik omicznych**

Study of *Trichoderma harzianum* extracellular
metabolites activity as natural elicitors, using omics
techniques

Praca doktorska

wykonana w Katedrze Mikrobiologii
Przemysłowej i Biotechnologii,
Instytutu Mikrobiologii, Biotechnologii i
Immunologii

Promotor:

- Dr hab. Przemysław Bernat

*Dziękuję swojemu Promotorowi,
dr hab. Przemysławowi Bernatowi prof. UŁ,
za poświęcony czas, cierpliwość i wyrozumiałość.*

*Serdeczne podziękowanie pracownikom KMPiB
za wszechstronną pomoc, miłą i życzliwą atmosferę.*

*Szczególne podziękowania dla Rodziny,
bez ich wsparcia ta praca by nie powstała.*

Spis treści

1. Finansowanie badań.....	2
2. Wykaz publikacji będących podstawą rozprawy doktorskiej.....	3
3. Dorobek naukowy.....	4
4. Streszczenie w języku polskim.....	7
5. Streszczenie w języku angielskim	9
6. Wprowadzenie	11
7. Cele pracy	13
8. Metodologia badań	14
9. Syntetyczne omówienie wyników przedstawionych w cyklu publikacji wchodzących w skład rozprawy doktorskiej.....	16
P-1 „Lipids, proteins and extracellular metabolites of <i>Trichoderma harzianum</i> modifications caused by 2,4-dichlorophenoxyacetic acid as a plant growth stimulator”.....	19
P-1 Supplementary material	30
P-2 “ <i>Trichoderma harzianum</i> metabolites disturb <i>Fusarium culmorum</i> metabolism: Metabolomic and proteomic studies"	39
P-2 Supplementary material	51
P-3 “Potential of <i>Trichoderma harzianum</i> and Its Metabolites to Protect Wheat Seedlings against <i>Fusarium culmorum</i> and 2,4-D.”.....	60
P-3 Supplementary material	82
10. Podsumowanie i stwierdzenia końcowe.....	99
12. Literatura uzupełniająca	102
13. Oświadczenie współautorów o udziale w publikacjach.....	105

1. Finansowanie badań

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2. Wykaz publikacji będących podstawą rozprawy doktorskiej

P-1:

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IF: 6,23; MNiSW: 100

P-2:

Mironenka J., Różalska S., Soboń A., Bernat P.*, 2021. “*Trichoderma harzianum* metabolites disturb *Fusarium culmorum* metabolism: Metabolomic and proteomic studies”. *Microbiological Research*, vol.249, 126770.

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IF: 5,415; MNiSW: 100

P-3:

Mironenka J., Różalska S., Bernat P.*, 2021. “Potential of *Trichoderma harzianum* and Its Metabolites to Protect Wheat Seedlings against *Fusarium culmorum* and 2,4-D.” *International Journal of Molecular Sciences*, vol. 22(23),13058.

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* Autorzy korespondujący

3. Dorobek naukowy

Publikacje

Szewczyk R., Różalska S., **Mironenka J.**, Bernat P., 2020. "Atrazine biodegradation by mycoinsecticide *Metarhizium robertsii*: Insights into its amino acids and lipids profile". Journal of Environmental Management, vol. 262, 1-9.

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Bernat P., Nykiel-Szymańska J., Gajewska E., Różalska S., Stolarek P., **Dackowa J.**, Słaba M. 2018 "Trichoderma harzianum diminished oxidative stress caused by 2,4-dichlorophenoxyacetic acid (2,4-D) in wheat, with insights from lipidomics." Journal of Plant Physiology.

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Monografie

Julia Mironenka, Przemysław Bernat (2021). Ogólnopolska Konferencja Doktorantów „Srodowisko – Roślina – Zwierzę – Produkt”. Monografia pod tytułem „Wpływ *Fusarium culmorum* na wytwarzanie wybranych zewnątrzkomórkowych metabolitów przez *Trichoderma harzianum*".

Udział w konferencjach naukowych

1. Julia Mironenka, Przemysław Bernat. IV Ogólnopolska Konferencja Naukowa „Pierwotne I Wtórne Metabolity Roślin I Grzybów” (15.VII.2021) „Wpływ zewnątrzkomórkowych metabolitów *Trichoderma harzianum* na syntezę zearalenonu *Fusarium culmorum* w podłożu płynnym”
2. Julia Mironenka, Przemysław Bernat, Sylwia Różalska. MycoRIse Up! 2021 (23-25.04.2021) " „Wpływ metabolitów *Trichoderma harzianum* na stres oksydacyjny *Fusarium culmorum*”
3. Julia Mironenka, Przemysław Bernat. Ogólnopolska Konferencja Doktorantów „Srodowisko – Roślina – Zwierzę – Produkt” (15.04.2021) "Zmiany w lipidomie płynnej hodowli *Trichoderma harzianum* w obecności kwasu 2,4-dichlorofenoksyoctowy”

4. Julia Mironenka, Przemysław Bernat. „Pierwotne I Wtórne Metabolity Roślin I Grzybów” (14.12.2020) „Wpływ metabolitów zewnątrzkomórkowych *Trichoderma harzianum* na wzrost i rozwój *Fusarium culmorum* na płytkach”
5. Julia Mironenka, Przemysław Bernat. “Metabolomics Circle 2020” (06.11.2020) “The influence of 2,4-D on the lipid peroxidation of *Trichoderma harzianum*”
6. Julia Mironenka, Przemysław Bernat. National Scientific Conference „Knowledge – Key to Success 2020” (18.01.2020) “Antibiotic peptides from *Trichoderma* species“
7. Przemysław Bernat, Justyna Nykiel-Szymańska, Ewa Gajewska, Sylwia Różalska, Julia Mironenka, Mirosława Słaba. International Symposium Microbe-assisted crop production (Micrope) (2-5.12.2019) "*Trichoderma harzianum* diminished oxidative stress caused by 2,4- dichlorophenoxyacetic acid (2,4-D) in wheat, with insights from lipidomics"
8. Julia Mironenka, Przemysław Bernat, Sylwia Różalska. 7th Central European Congress of Life Sciences (23-25.09.2019) „Effect of 2,4-D on the permeability of the *Trichoderma harzianum* cell membrane”
9. Julia Mironenka, Przemysław Bernat. Intercollegiate Biotechnology Symposium „Symbioza” (17-19.05.2019) „Multidirectional MTBE extracion”
10. Julia Mironenka, Przemysław Bernat. MycoRise Up! Młodzi w mykologii (12-13.04.2019) "Ograniczenie toksycznego wpływu 2,4-D na pszenicę przez *Trichoderma harzianum*”
11. Julia Mironenka, Przemysław Bernat. National Scientific Conference for PhD Students (02.03.2019) “Effect of *Trichoderma harzianum* extract on the growth and production of *Fusarium culmorum* mycotoxins”
12. Julia Mironenka, Przemysław Bernat, Sylwia Różalska, Rafał Szewczyk. III Konferencja Doktorantów Nauk Przyrodniczych (25-28.06.2019) "Wpływ atrazyny na błony biologiczne *Metarhizium robertsii*"
13. Julia Mironenka, Przemysław Bernat. National Scientific Conference „Knowledge – Key to Success 2019” (19.01.2019) „Study of the wheat lipid profile interacting with *Trichoderma harzianum*, in the presence of the phenoxyacetic herbicide”.

Kursy

Sample Preparation for MS Bioanalysis: Theory, Practice and Applications
(28 September- 1 October 2021), Bydgoszcz, Poland.

4. Streszczenie w języku polskim

Mikroskopowe grzyby strzępkowe z rodzaju *Trichoderma* znajdują szerokie zastosowanie w gospodarce człowieka, w tym rolnictwie. Zaobserwowano, że wybrane gatunki z tego rodzaju mogą wpływać pozytywnie na wzrost roślin i hamować aktywność fitopatogenów popularnie uprawianych zbóż.

Uprawa pszenicy często jest narażona na oddziaływanie abiotycznych i biotycznych czynników stresowych. W celu zwalczania chwastów poddaje się ją oddziaływaniu chemicznych środków ochrony roślin. Do biotycznych czynników stresowych należy w szczególności aktywność grzybów mikroskopowych z rodzaju *Fusarium*, które mogą wywoływać takie choroby roślin jak fuzariotyczna zgnilizna korony czy fuzarioza kłosów.

Niniejsza praca doktorska dotyczy możliwości wykorzystania grzybów z gatunku *Trichoderma harzianum*, jako potencjalny czynnik kontroli biologicznej, chroniący pszenicę przed stresem biotycznym i abiotycznym oraz wspomagający wzrost rośliny we wczesnych stadiach kiełkowania.

W pierwszym etapie pracy oceniono wpływ wybranego do badań herbicydu – kwasu 2,4-dichlorofenoksyoctowego (2,4-D) na wzrost i rozwój *T. harzianum* w hodowli płynnej. Uzyskane wyniki wykazały brak istotnego oddziaływania tego herbicydu na wzrost drobnoustroju. Za pomocą techniki rozdzału elektroforetycznego białek (1-D), zidentyfikowano białka biorące udział w reakcji oksydoredukcyjnej. Stosując technikę mikroskopii fluorescencyjnej w hodowli z dodanym 2,4-D stwierdzono wzrost poziomu reaktywnych form tlenu RFT w fazie wzrostu wykładniczego, natomiast w próbie kontrolnej nie zaobserwowano ich obecności. Otrzymane wyniki ściśle korelowały z profilami oksylipin i fosfolipidów wskazujących na zachodzący stres w grzybni *T. harzianum*. Wykonano także oznaczenia zawartości metabolitów *T. harzianum* – kwasu harzianowego i T22-azofilonu, wykazano, że dodanie 2,4-D powodowało zahamowanie produkcji metabolitów w 24h. Powyższe analizy przeprowadzono z zastosowaniem techniki LC-MS/MS.

W kolejnym etapie pracy zbadano wpływ zewnątrzkomórkowych metabolitów *T. harzianum* na wzrost i rozwój *Fusarium culmorum* w hodowli stałej i hodowli płynnej. Uzyskane z hodowli na podłożu stałym wyniki wykazały, że metabolity *Trichoderma* wpływają na zahamowanie wzrostu grzybni (ok. 85%),

a także na zdolność do zarodnikowania. Odnotowano różnice w produkcji barwników naftochinonowych w hodowli kontrolnej - przed 96h i po 168h, natomiast w hodowli badanej 120h a 168h. Stosując technikę LC-MS/MS oznaczono stężenie syntetyzowanej przez wybrany szczep mykotoksyny – zearalenonu, w 120h zauważono 10-krotnie niższe wartości w hodowli badanej w stosunku do kontroli. Przeprowadzono również analizę porównawczą profilu białkowego grzybni stosując technikę 2-D. W hodowli badanej z dodanym ekstraktem z *T. harzianum* zidentyfikowano niezależną od GSH glioksalazę HSP31, która jest zaangażowana w reakcje obronne przed RFT. Technika LC-MS/MS oznaczono mniejsze ilości GSH w całym okresie hodowli w próbie badanej. Za pomocą techniki spektrofotometrycznej oznaczono enzymy katalazę (CAT) i dysmutazę ponadtlenkową (SOD), biorące udział w zwalczaniu RFT. Uzyskane wyniki potwierdziły niższy poziom aktywnej SOD w kontroli i całkowity brak CAT.

W trzecim etapie pracy doktorskiej oceniono zdolności metabolitów *Trichoderma* do wspomagania wzrostu pszenicy w obecności czynników stresowych. Udowodniono, że dodane metabolity na równi z zarodnikami *T. harzianum* przyczyniają się do szybszego kiełkowania pszenicy, a także wspomagają wzrost korzeni w układach z dodanym herbicydem. Aby ocenić poziom stresu zachodzącego w roślinie, techniką LC-MS/MS oznaczono zawartość kwasu jasmonowego, którego niższe ilości odnotowano zarówno w układach z czynnikami stresowymi i dodanymi zarodnikami, jak i czynnikami stresowymi oraz metabolitami *Trichoderma*. Na podstawie analizy porównawczej 2-D oznaczono różnice w profilach białek izolowanych z pędów i korzeni pszenicy. Zidentyfikowano wyraźny wpływ czynników stresowych na zdolności fotosyntezujące. Powyższe wyniki wykazywały dużą zbieżność z przeprowadzonym pomiarem ilości chlorofilu w pędach rośliny. Stwierdzono jego niższy poziom we wspomnianych układach, natomiast w obecności *T. harzianum* obserwowano wyższe ilości barwnika, co może świadczyć o obniżaniu toksycznego oddziaływanie herbicydu i fitopatogenu.

Przeprowadzone badania wskazują na zdolność zarodników *T. harzianum* jak i otrzymanego ekstraktu do wspomagania wzrostu pszenicy oraz na możliwość ich wykorzystania w celu niwelacji negatywnego oddziaływania biotycznych i abiotycznych czynników stresowych na roślinę.

5. Streszczenie w języku angielskim

Microscopic filamentous fungi belonging to *Trichoderma* are widely used in agriculture. It has been observed that selected species of this genus may positively affect plant growth and inhibit the activity of phytopathogens in popularly cultivated cereals.

The cultivation of crops like wheat is often exposed to both abiotic and biotic stressors. Herbicides are used in order to control weeds. Biotic stress factors include, in particular, activity of microscopic fungi of the genus *Fusarium*, which can cause plant diseases such as *Fusarium crown rot* (FCR) or *Fusarium head blight* (FHB).

The research subject of this doctoral thesis is possibility of using *Trichoderma harzianum* fungi as a potential biological control factor, which can protect wheat against biotic and abiotic stress and support plant growth in the early stages of germination.

In the pursuit of the first goal of the research, influence of the selected for the research herbicide (2,4-dichlorophenoxyacetic acid (2,4-D)) on the growth and development of *T. harzianum* in liquid culture was assessed. Obtained results showed that the addition of the herbicide did not significantly affect the growth of the microorganism. Content of 2,4-D has been determined after 5 days of cultivation using the technique of liquid chromatography coupled with tandem mass spectrometry (LC-MS / MS). Using the protein electrophoretic separation technique (1-D), proteins involved in the redox reaction have been identified. Using the technique of fluorescence microscopy an increase of reactive oxygen species (ROS) level was found in the exponential growth phase in the studied sample; while in the control, presence of ROS has not been observed. Obtained results closely correlated with the study of oxylipins and phospholipids profiles which indicate the oxidative stress taking place in the *T. harzianum* mycelium. The content of *T. harzianum* metabolites - harzianic acid and T22-azophyllone have been also performed. Addition of 2,4-D inhibited the production of metabolites in 24 hours. The study have been performed using the LC-MS/MS technique

In the next stage of the work, influence of extracellular metabolites of *T. harzianum* on the growth and development of *Fusarium culmorum* in solid and liquid culture has been investigated. *Trichoderma* metabolites added to the solid medium inhibit of mycelial growth (approx. 85%) and the sporulation ability.

Differences have been also noticed in *F. culmorum* pigment production. Presence of naphthoquinone pigments in the culture medium depending on the duration of the culture (in the control culture - the highest concentration was noticed before 96h and after 168h, in the tested culture between 120h and 168h). Concentration of the main mycotoxin - zearalenone, synthesized by the selected strain, has been determined using the LC-MS/MS technique. At 120h, 10 times lower concentration has been noticed in the test culture compared to the control. A comparative analysis of the protein profile of the mycelium has been performed using the 2-D technique. GSH-independent glyoxase HSP31, which is involved in protective responses against ROS, has been identified in culture with added *T. harzianum* extract. Lower amounts of GSH have been determined in the whole cultivation period in the studied culture, using the LC-MS/MS technique. Using the spectrophotometric technique, enzymes catalase (CAT) and superoxide dismutase (SOD), involved in the scavenging mechanism of ROS, have been determined. Obtained results confirmed lower level of active SOD in the control and complete absence of CAT.

In the third part of the doctoral study, ability of *Trichoderma* metabolites to support wheat growth in the presence of stress factors has been assessed. It has been proven that added metabolites, along with *T. harzianum* spores, contribute to faster germination of wheat, and also support root growth in systems with the added herbicide. Using the LC-MS/MS technique, jasmonic acid, in order to assess the level of stress occurring in the plant, has been determined. The lower amount of the hormone has been identified both in systems with stress factors and added spores, as well as stress factors and *Trichoderma* metabolites. Based on a 2-D comparative analysis, differences in the profiles of proteins isolated from wheat shoots and roots have been determined. A clear influence of stress factors on photosynthetic capacity has been identified. obtained results showed a great convergence with the study of chlorophyll content in the wheat shoots, which level was lower in earlier mentioned systems, while the addition of *T. harzianum* picked up it amounts, which may indicate elimination of the toxic effect of the herbicide and phytopathogen.

Conducted studies indicate the ability of *T. harzianum* spores and obtained extracts to support wheat growth and possibility of using them to eliminate negative impact of the biotic and abiotic stress factors on the plant.

6. Wprowadzenie

Rodzaj *Trichoderma* obejmuje dużą liczbę gatunków prowadzących głównie saprotroficzny i mykopasożytniczy tryb życia [1]. Wiele z tych drobnoustrojów posiada zdolność do wytwarzania licznych enzymów zewnątrzkomórkowych, które znalazły zastosowanie w gospodarce człowieka.

Wśród grzybów należących do tego rodzaju wyróżnia się także gatunki o właściwościach antagonistycznych wobec patogenów roślin. Zaobserwowano, że te pożyteczne drobnoustroje mogą oddziaływać na fitopatogeny poprzez lizę enzymatyczną, wytwarzanie antybiotyków oraz rywalizację o przestrzeń i substraty odżywcze [2].

Spośród omawianego rodzaju gatunek *T. harzianum* jest dobrze znany ze swojego korzystnego wpływu na rośliny. Stwierdzono, że może przyspieszać wzrost rzodkiewnika pospolitego (*Arabidopsis thaliana*), kapusty ogrodowej (*Brassica oleracea*) a także wpływać na rozwój i kiełkowanie korzeni *Brassica napus* [3].

Ekstrakty uzyskane ze szczepów *T. harzianum* E45 i ET45, wykazały aktywność przeciwdrobnoustrojową wobec szczepów *Staphylococcus pseudointermedius*. Jako główny związek odpowiedzialny za aktywność biologiczną tych ekstraktów uznano kwas harzianowy [4], który wyizolowany ze szczepu M10 wykazywał także aktywność przeciwdrobnoustrojową przeciwko *Pythium irregulare*, *Sclerotinia sclerotiorum*, i *Rhizoctonia solani* [5].

Pszenica zajmuje czołowe miejsce w światowej produkcji zbóż. Jej uprawy są często narażone na różne stresy abiotyczne i biotyczne. Narażenie na stres abiotyczny roślin może być związane z działaniem związków chwastobójczych aplikowanych na uprawy. Jednym z najpopularniejszych herbicydów są preparaty zawierające kwas 2,4-dichlorofenoksyoctowy (2,4-D). Pestycydy te charakteryzuje niska cena, wysoka efektywność i selektywność. 2,4-D naśladuje działanie hormonów roślinnych i nazywany jest syntetyczną auksyną. Zaaplikowanie 2,4-D powoduje niekontrolowany wzrost, a w kolejnym etapie następuje obumieranie roślin, podatnych na jego działanie. Uprawy pszenicy, ryżu, jęczmienia są również narażone

na negatywne działanie 2,4-D, którego pozostałości mogą być wchłaniane z gleby przez rośliny.

Uprawy pszenicy są narażone na stres biotyczny, poprzez obecność mikroorganizmów bytujących w glebie. Popularnymi patogenami pszenicy są grzyby z rodzaju *Fusarium*, odpowiedzialne m. in. za fuzariotyczną zgniliznę korony (FCR - ang. *Fusarium crown rot*) i fuzariozę kłosów (FHB - ang. *Fusarium head blight*). Są to dwie poważne choroby grzybowe, prowadzące do znacznych strat plonów i obniżenia cen rynkowych pszenicy z powodu złej jakości ziarna.

Do niebezpiecznych patogenów zbóż zalicza się w szczególności grzyby z gatunków *F. graminearum* i *F. culmorum*, a ich metabolity wtórne takiej jak deoksyniwalenol (DON), zearalenon (ZEA) i fumozyna B uważa się za jedne z najgroźniejszych mykotoksyn [6].

Dotychczas nie znaleziono skutecznych środków ochrony pszenicy przeciwko chorobom wywołanym przez *Fusarium*. Mając na uwadze duże zagrożenie, jakie stwarzają fitopatogeny i poszukując przyjaznych środowisku sposobów na walkę z nimi, zwrócono uwagę na stosowanie czynników kontroli biologicznej (BCA – ang. *Biological Control Agents*) opartej o użycie żywych mikroorganizmów, lub ich metabolitów do kontroli szkodników w uprawach.

Niniejsza praca doktorska dotyczy możliwości wykorzystania grzybów z gatunku *Trichoderma harzianum*, jako potencjalny czynnik kontroli biologicznej, chroniący pszenicę przed stresem biotycznym i abiotycznym oraz wspomagający wzrost rośliny we wczesnych stadiach kiełkowania. Do badań wykorzystano szczepy grzybowe:

T. harzianum IM0961 z Kolekcji Katedry Mikrobiologii Przemysłowej i Biotechnologii (UŁ), szczep KKP534 z Kolekcji Kultur Drobnoustrojów Przemysłowych (Warszawa) oraz szczep *F. culmorum* DSM 1094 zakupiony z Niemieckiej kolekcji mikroorganizmów i kultur komórkowych GmbH (DSMZ); jako model roślinny wybrano nasiona *Triticum aestivum* L. (cv. Zyta) – pozyskane ze Stacji Hodowli Roślin Strzelce Sp. z o.o. Grupa IHAR.

7. Cele pracy

1. Oznaczenie wpływu kwasu 2,4-dichlorofenoksyoctowego (2,4-D) na wzrost i syntezę metabolitów wtórnych przez *T. harzianum* w hodowli płynnej.
2. Określenie wpływu metabolitów zewnątrzkomórkowych *T. harzianum* na wzrost *F. culmorum* i produkcję metabolitów wtórnych w podłożu stałym oraz w hodowli płynnej.
3. Zbadanie efektu układu zintegrowanego na kiełkowanie nasion pszenicy: wpływ stresu abiotycznego w postaci dodanego herbicydu (2,4-D), stresu biotycznego w postaci dodanego patogenu (*F. culmorum*) oraz potencjalnego elicytora – *T. harzianum*.
4. Oznaczenie możliwości zastosowania ekstraktów z płynu pohodowlanego *T. harzianum*, jako potencjalnych preparatów wspomagających wzrost pszenicy w obecności stresu biotycznego i abiotycznego.

8. Metodologia badań

a. Techniki wykorzystywane podczas realizacji pracy doktorskiej

- **LC-MS/MS (Agilent 1200 HPLC system (USA); 3200, 4500 Q-TRAP mass spectrometer (Sciex, USA))** Technika rozdzielania chromatograficznego materiału sprzężona ze spektrometrią mas – identyfikacją związków na podstawie masy naładowanej cząsteczki do ładunku. Technika wykorzystana do analizy ilościowej metabolitów (P-1), mykotoksyn (P-2, P-3), fosfolipidów (P-1, P-2, P-3), oksylipin (P-1, P-3), glutationu (P-2), pigmentów (P-2), stężenia herbicydu (P-1, P-3).
- **Mikroskopia fluorescencyjna i konfokalna (Zeiss, Germany)** Technika obrazowania optycznego z oznaczeniem zmian na podstawie kontrastu rejestrowanego obrazu. Zastosowano w P-1 w celu oznaczenia uszkodzenia błony komórkowej, P-1 i P-2 do oznaczenia anionorodników.
- **FLUOstar Omega spectrofluorometer (BMG Labtech, Germany)** Za pomocą pomiaru natężenia fluorescencji na płytkach, (P-1) przeprowadzono pomiary przepuszczalności błony komórkowej oraz użyto do pomiarów stężenia wyizolowanych białek (P-1, P-2, P-3).
- **Elektroforeza 1-D oraz 2-D (Bio-Rad, Germany)** Rozdział na żelu agarozowym za pomocą prądu elektrycznego na podstawie wielkości, kształtu i ładunku elektrycznego białek. Technikę 1D zastosowano w P-1 do rozdzielania zewnątrzkomórkowych białek; technikę 2D wykorzystano w P-2 do rozdzielania wewnątrzkomórkowych białek; w P-3 do rozdzielania białek pędów i korzeni.
- **Spektrometria mas MALDI-TOF/TOF (AbSciex 5800 TOF/TOF system, USA)** Analiza peptydów na podstawie ich masy do ładunku. Technikę wykorzystano w P-1, P-2, P-3.
- **Chlorophyll Content Meter CCM-300 (Opti-Sciences (USA))** urządzenie pozwalająca na określenie zawartości chlorofilu na podstawie współczynnika emisji fluorescencji. Wykorzystano w P-3 do pomiaru stężenia chlorofilu w pędach.

b. Oprogramowanie

- **Image Lab (Bio-Rad, Germany)** oprogramowanie do akwizycji i analizy żelu 1D. Wykorzystane zostało w P-1 do analizy profilu zewnątrzkomórkowych białek.
- **Image Master 2D Platinum (GE Healthcare (USA))** oprogramowanie do analizy żeli 2D, pozwala na identyfikację różnic pomiędzy żelami oraz na definiowanie wielokrotnego dopasowania. Wykorzystano w P-2 w celu zróżnicowania intensywności spotów.
- **SameSpot (the United Kingdom)** oprogramowanie dające obiektywne wyniki dla zróżnicowanej ekspresji nienaruszonych białek przy użyciu żeli 2D, pozwala na szybką analizę dużej ilości prób, zapewniając analizę statystyczną. Wykorzystano w P-3 do identyfikacji różnic w żelach pochodzących z 12 układów.
- **Protein Pilot v4.5 (Sciex (USA))** oprogramowanie do identyfikacji białek. Białka pod działaniem enzymu – trypsyny, ulegają proteolizie. Po otrzymaniu widm masowych po zeskanowaniu przez MALDI TOF/TOF, na podstawie algorytmu oprogramowanie dopasowuje otrzymane widma fragmentacyjne peptydów do bazy danych **Mascot v2.4 (Matrix Science, the United Kingdom)**. Wynik może być prawidłowy po osiągnięciu pewnego progu Score (dopasowania). Wykorzystano w P-1, P-2, P-3 w celu oznaczenia wybranych białek.
- **STATISTICA c.13.3 (USA)** oprogramowanie analityczne, zapewniające analizę statystyczną danych. Ocenę statystycznej istotności przeprowadzono z wykorzystaniem analizy wariancji testem post hoc Tukeya, Test należy do jednoetapowych procedur wielokrotnych porównań otrzymanych wyników, w celu znalezienia różnic statystycznie istotnych. Wykorzystano w P-1, P-2, P-3, wyniki uznano za istotne przy $p < 0,05$.

9. Syntetyczne omówienie wyników przedstawionych w cyklu publikacji wchodzących w skład rozprawy doktorskiej

Pierwszym etapem pracy doktorskiej, było oznaczenie skutków zaaplikowania herbicydu - 2,4-D, na wzrost *T. harzianum* w hodowli płynnej a także na produkcję metabolitów. Szczep ten jest znany z wytwarzania licznych metabolitów wtórnych, które znalazły zastosowanie w różnych działach gospodarki człowieka [7].

We wstępnych badaniach niniejszej pracy doktorskiej sprawdzono jak różne stężenia 2,4-D (w zakresie 10 – 200 mg/l) wpływają na wzrost *T. harzianum* [dane niepublikowane]. Dodając do układu herbicyd w stężeniu 100 mg/l, w ciągu 5 dni hodowli w kontrolowanych warunkach (wyrząsanie 160 rpm, 28 °C) zaobserwowano lekkie opóźnienie oraz zahamowanie wzrostu *T. harzianum*. Zaaplikowane stężenie herbicydu w badanej hodowli powodowało zmianę zabarwienia płynu pohodowlanego, w porównaniu do kontroli biotycznej. Za pomocą techniki LC-MS/MS zmierzono ilość herbicydu w hodowlach grzybowych – w stosunku do ilości herbicydu w kontroli abiotycznej (przyjęto, jako 100%) stwierdzono obecność 96% herbicydu. Nie wykryto również w płynie pohodowlanym potencjalnych metabolitów degradacji 2,4-D. Na podstawie zebranych wyników, do dalszych badań postanowiono wykorzystać punkty pomiarowe z fazy wykładniczego wzrostu (24h) i fazy stacjonarnej (96h) krzywej wzrostu *T. harzianum*.

Na podstawie wcześniej przeprowadzonych badań w Katedrze Mikrobiologii Przemysłowej i Biotechnologii UŁ oraz danych literaturowych można przypuszczać, że działanie 2,4-D jest związane z oddziaływaniem na błony komórkowe mikroorganizmów [8]. Dlatego postanowiono ocenić jego wpływ na fosfolipidy grzybowe, (które stanowią istotny element budulcowy błon biologicznych) za pomocą techniki chromatografii cieczowej sprzężonej ze spektrometrią mas i przepuszczalność błony komórkowej *T. harzianum* z użyciem techniki mikroskopii fluorescencyjnej. W grzybniach hodowanych w obecności herbicydu w fazie wykładniczej odnotowano statystycznie istotne zwiększenie ilości fosfolipidów z grupy fosfatydyloetanolamin (PE – ang. *phosphatidylethanolamine*) i mniejsze wartości fosfatydylocholin (PC – ang. *phosphatidylcholine*) w porównaniu do układu kontrolnego. Ponieważ PC mogą ulegać metylacji do PE z udziałem metylotransferazy fosfolipidowej [9],

można przypuszczać, że w obecności 2,4-D w 24h nastąpiło zaburzenie procesu metylacji. Obecność 2,4-D miała wpływ także na wyższy poziom kwasu fosfatydowego (PA – ang. *phosphatidic acid*), które w układach niepoddanych czynnikom stresowym występują w bardzo małych ilościach w błonie komórkowej, a ich podwyższony poziom może świadczyć o zaburzeniu równowagi wewnętrznej drobnoustroju [10]. W celu potwierdzenia oznaczonych zmian w profilu fosfolipidów, które mają wpływ na przepuszczalność błony komórkowej, w kolejnym badaniu podjęto próby oznaczenia uszkodzeń błony komórkowej, za pomocą fluorescencyjnego odczynnika - jodku propidyny, który po wnikięciu do komórki wiąże się z DNA i może być wykryty za pomocą mikroskopu fluorescencyjnego. Wyniki potwierdziły wzrost przepuszczalności błony komórkowej w próbach badanych (1,5 i 3 U/mg suchej masy), w badanych fazach wzrostu w porównaniu do kontroli (0,1 i 0,7 U/mg suchej masy).

Istotnym celem pierwszego etapu badań było oznaczenie wpływu dodanego herbicydu na produkcję i wytwarzanie metabolitów zewnątrzkomórkowych *T. harzianum*. Stosując technikę LC–MS/MS, potwierdzono obecność związków, opisywanych przez innych badaczy, charakterystycznych dla szczepów *T. harzianum*: kwasu harzianowego i t22-azafilonu [11, 12]. Wykazano, że związki te wykazują aktywność antybiotykową *in-vitro* przeciwko *R. solani*, *Pythium ultima* lub *Sclerotinia sclerotiorum* [5]. Podczas wykładniczej fazy wzrostu badanego drobnoustroju ilości kwasu harzianowego i t22-azafilonu były czterokrotnie niższe w hodowli traktowanej 2,4-D w porównaniu z hodowlą kontrolną, w kolejnej fazie wzrostu ilość kwasu harzianowego była trzykrotnie mniejsza.

Dodatkowo w pierwszym etapie pracy postanowiono ocenić zmiany zachodzące w zewnątrzkomórkowej produkcji białek przez *T. harzianum* w obecności 2,4-D. W celu rozdziału białek zastosowano technikę jednokierunkowej elektroforezy, z identyfikacją białek po proteolizie za pomocą MALDI TOF/TOF. Wyniki dopasowano do bazy Mascot v2.4 (Matrix Science, UK) za pomocą oprogramowania ProteinPilot. v4.5 (Sciex, USA). We wszystkich próbach oznaczono obecność oksydazy cukrowej 1,4-laktonowej; dehydrogenaza zawierająca FAD/FMN; domeny wiążącej FAD – białka biorące udział w odpowiedzi oksydoredukcyjnej. Przeprowadzona analiza proteomiczna nie umożliwiała oceny ilościowej zachodzących zmian w reakcji utleniania, dlatego stosując testy NBT, H₂DCFDA

i DAB zmierzono poziom O_2^- , $NO\bullet$ i H_2O_2 . Otrzymane wyniki wskazywały na większą ilość H_2O_2 w hodowli traktowanej herbicydem w porównaniu do kontroli. Stwierdzono również wzrost wewnątrzkomórkowego poziomu anionu nadadtlenkowego w hodowli z 2,4-D i $NO\bullet$ w fazie wzrostu wykładniczego, w próbie kontrolnej nie stwierdzono obecności tych reaktywnych form tlenu (RFT). Obserwując istotne statystycznie zmiany dla powyższych analiz postanowiono wykonać także oznaczenia oksylipin, będących produktami utlenienia kwasów tłuszczowych. W próbach badanych oznaczono podwyższony poziom hydroksylowanego kwasu linolowego – 9-HODE i 13-HODE, – które są uważane za markery peroksydacji lipidów [13]. Uzyskane wyniki potwierdziły, że 2,4-D indukuje stres oksydacyjny w hodowli płynnej *T. harzianum*.

Pozostałe białka oznaczone w danym etapie nawiązywały do wyników otrzymanych z analizy fosfolipidów (białko podobne do fosfolipazy B z grzybów; domena katalityczna lizofosfolipazy; fosfolipaza A2), i wskazywały na zachodzącą reakcję obronną w układzie z herbicydem (cerato-platanin).

Podsumowując pierwszy etap badań odnotowano, że obecność herbicydu w hodowli płynnej miała wpływ na rozwój i wytwarzanie metabolitów wtórnych *T. harzianum*. W przeprowadzonej części pracy doktorskiej zastosowano 6 technik analitycznych (m.in. chromatografia cieczowa ze sprzężoną spektrometrią mas, mikroskopia fluorescencyjna, metody spektrofotometryczne, metoda jednokierunkowej elektroforezy z identyfikacją białek metodą MALDI-TOF/TOF), a łącznie użyto trzynastu różnych metod. Po zakończeniu pierwszej części pracy doktorskiej wyniki opublikowano w czasopiśmie *Ecotoxicology and Environmental Safety* (IF: 6,23; MNIŚW: 100) pod tytułem „Lipids, proteins and extracellular metabolites of *Trichoderma harzianum* modifications caused by 2,4-dichlorophenoxyacetic acid as a plant growth stimulator”.

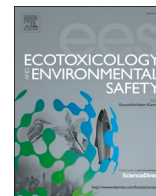
P-1

„Lipids, proteins and extracellular metabolites of *Trichoderma harzianum* modifications caused by 2,4-dichlorophenoxyacetic acid as a plant growth stimulator”.

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Lipids, proteins and extracellular metabolites of *Trichoderma harzianum* modifications caused by 2,4-dichlorophenoxyacetic acid as a plant growth stimulator

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ABSTRACT

Strains of *Trichoderma harzianum* are well-known producers of bioactive secondary metabolites and have a beneficial effect on plants. However, to the best of our knowledge, the effect of the commonly used pesticides on the activity of this fungus is not yet investigated. Therefore, in the present study, the effect of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) on the lipidome and selected extracellular compounds synthesized by *T. harzianum* IM 0961 was examined. It was observed that the herbicide 2,4-D caused changes in the lipid composition of the mycelium and that the herbicide exhibited lipophilic properties. In addition, the herbicide disturbed the phosphatidylcholine (PC)/phosphatidylethanolamine (PE) ratio and increased membrane permeability. The higher amount of cardiolipin CL 72:7 and the lower amount of CL 72:8 could have been associated with a decreased ratio of 18:2 and 18:1 fatty acids in the herbicide-treated samples. Moreover, in the presence of 2,4-D, an increased lipid peroxidation (twofold), as well as a higher content of oxylipin (9-HODE and 13-HODE) and phosphatidic acid (PA), was noted, confirming that 2,4-D induced lipid peroxidation in the mycelium. The herbicide also exerted its toxic effect on the production of 14-aminoacid peptaibols and two compounds, harzianic acid and t22-azaphilone, exhibiting antibiotic and plant growth-promoting activity. During proteomic analysis, the synthesis of some proteins, such as calcineurin-like phosphoesterase metallophosphatases (MPPs), which modulate the properties of cell walls, was found to be inhibited by the herbicide. These presented findings may be of significant value in understanding the effect of 2,4-D on the activity of *T. harzianum*.

1. Introduction

Trichoderma is one of the most studied genera of fungi. The reason for the interest in this fungal genus is its practical and ecological significance. It is widely used to protect plants against pathogens and increase their resistance against abiotic stress (Ortega-Garcia et al., 2015). Since the 1930s, *Trichoderma* has been used for controlling plant diseases (Saba et al., 2012). Due to its ability to parasitize phytopathogenic fungi and enhance immunity, *Trichoderma* is used as a commercial biofungicide in agriculture (Zeilinger et al., 2016).

Among the various activities of *Trichoderma*, the most widely studied is the ability of this fungal genus to produce numerous extracellular small-molecule metabolites. Currently, about 390 nonvolatile compounds (Li et al., 2019) and 140 volatile metabolites, also known as volatile organic compounds (VOCs), have been identified to be

produced by these microorganisms (Lee et al., 2016). Some of the selected metabolites of *Trichoderma* have also been found to elicit systemic resistance in plants (Contreras-Cornejo et al., 2016).

In our previous study, we described that a *Trichoderma harzianum* strain was capable of partly alleviating the toxic effect of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) on wheat seedlings (Bernat et al., 2018a). This strain was also well known for its beneficial effect on plants and found to increase the productivity of *Arabidopsis thaliana*, *Brassica oleracea*, and *Brassica napus* (Poveda et al., 2019), as well as leading to the early root germination of *Rhizoctonia solani* (Manganiello et al., 2018).

However, to the best of our knowledge, the effect of the commonly used pesticides on the activity of *T. harzianum* is not yet explained. Recently, a study described the mechanisms of tolerance exhibited by *Trichoderma asperellum* TJ01 toward the organophosphorus pesticide

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dichlorvos (Wu et al., 2018), but no study has investigated how one of the most popular herbicides, 2,4-D, influences the activity of *T. harzianum* so far.

Therefore, the aim of this study was to evaluate the effect of 2,4-D on *T. harzianum*. The liposolubility of 2,4-D is believed to affect the function of the cell membrane (Viegas et al., 2005) and therefore play an important role in its mechanisms of toxicity. Taking this fact into account, we analyzed the lipid composition of *T. harzianum* using liquid chromatography–mass spectrometry (LC–MS/MS). Furthermore, the content of oxylipin (a bioactive lipid metabolite of *T. harzianum*) was estimated, and the cell membrane permeability and production of reactive oxygen species (ROS) were studied. These results were supplemented by proteomic analysis of the extracellular proteins of *T. harzianum*, and an investigation of the relationship between the lipid profile, oxidative stress, and protein profile of the species. *Trichoderma harzianum* is known to produce compounds with antibiotic properties and promote plant growth. Therefore, in the next part of our study, we identified some of these compounds and observed the impact of 2,4-D on their production using LC–MS/MS and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) technique.

2. Materials and methods

2.1. Reagents

The herbicide 2,4-D and 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) were purchased from Sigma-Aldrich (Germany). Phospholipid standards were procured from Avanti Polar Lipids (USA), and oxylipin standards from Cayman Chemical (USA). All the materials used in the proteomic analysis were purchased from Bio-Rad, Promega (trypsin), and Sigma-Aldrich (6500- to 200,000-Da mass marker). Other solvents were acquired from Avantor Performance Materials (Poland). The stock solutions of 2,4-D were prepared at a concentration of 5 mg/mL in ethanol.

2.2. Strain and growth conditions

The fungal strain *T. harzianum* IM 0961 was obtained from the Department of Industrial Microbiology and Biotechnology, University of Lodz. Spores isolated from the 7-day cultures grown on ZT agar slants (g/L: glucose, 4; Difco yeast extract, 4; agar, 25; malt extract 6° Balling (BLG) up to 1 L [1° BLG = 1 g of soluble substances extracted from the grain per 100 mL of malt extract]; pH 7.0) were used in the study.

The obtained fungal spores inoculated in 20 mL Sabouraud dextrose broth medium (Difco) added in 100 mL Erlenmeyer flasks (Bernat et al., 2018b). The spores were cultured on a rotary shaker (160 rpm) for 48 h at 28 °C. Then, 2 mL of preculture was introduced into Sabouraud medium supplemented with 100 mg/L 2,4-D or the control culture medium without the herbicide. All cultures were incubated on a rotary shaker (160 rpm) at 28 °C. After 24 h and 96 h of incubation, the biomass was exudated using a filter paper and quantified by the method described by Bernat et al. (2013).

All experiments were conducted in the exponential (24 h) and early stationary (96 h) growth phases in the case of both control and 2,4-D-treated samples.

2.3. Analysis of 2,4-D

The activity of 2,4-D in the samples was determined using the methods described in our previous work (Nykiel-Szymańska et al., 2018).

2.4. Phospholipids determination

Phospholipids of *T. harzianum* were extracted following our previous method (Bernat et al., 2018b) but with some modifications.

Briefly, 50 mg of fungal biomass separated on the filter paper was washed with distilled water and transferred into a 2-mL Eppendorf tube containing glass beads, 0.66 mL methanol, and 0.33 mL chloroform. The biomass was homogenized for 1 min (each cycle for 20 s) with a homogenizer (FastPrep-24, MP Biomedicals). The mixture was transferred to another Eppendorf tube, and 0.2 mL of 0.9% saline was added. Then, the sample was vortexed and centrifuged at 2000 × g for 5 min. After centrifugation, the organic lower layer was collected, evaporated under reduced pressure, and stored at −20 °C until it was used for phospholipid analysis by LC–MS/MS (Agilent 1200 HPLC system (Agilent, USA) with 4500 Q-TRAP mass spectrometer (Sciex, USA)).

The obtained lipid extract was first fractionated using the Agilent 1200 HPLC system (USA). For this, 10 µL of the extract was injected into a Kinetex C18 column (50 mm × 2.1 mm, particle size: 5 µm; Phenomenex, USA) at a flow rate of 500 µL min^{−1}. The column temperature was maintained at 40 °C. The mobile phases used were water (A) and methanol (B), both of which consisted of 5 mM ammonium formate. The solvent elution was initiated from 70% B and then increased to 95% B over 1.25 min and maintained at 95% B for 6 min before returning to the initial solvent composition over 3 min. The mass spectrometric analysis was carried out using the 4500 Q-TRAP mass spectrometer (Sciex, USA), equipped with an electrospray ionization (ESI) source, under the following conditions: spray voltage −4500 V, curtain gas 25 psi, nebulizer gas 50 psi, turbo gas 60 psi, and ion source temperature 600 °C. Data analysis was performed using the Analyst™ v1.6.2 software (Sciex, USA). The qualitative and quantitative analyses were carried out according to the methods described in our previous works (Bernat et al., 2014, Bernat et al., 2018b).

2.5. Other lipids determinations

The obtained lipid extract (prepared in the same way as phospholipids extraction) was used for ergosterol, triacylglycerols (TAGs) and sphingolipids determination. Analyses of these lipids were performed according to the procedure described in our previous paper (Bernat et al., 2018b).

2.6. Oxylipin analysis

For the analysis of oxylipin, 50 mg of wet biomass was harvested, transferred to a 2-mL Eppendorf tube containing glass beads, and frozen in liquid nitrogen. Then, the samples were homogenized using the FastPrep-24 instrument (MP Biomedicals) at 5 m s^{−1} for 20 s (total time 1 min). Oxylipin was extracted from the samples according to the method of Salem et al. (2016) with some modifications. Briefly, 1 mL of a mixture of methyl tert-butyl ether with methanol (3:1, v/v) was added to the homogenized samples and the samples were vortexed for 1 min. Then, 650 µL of a mixture of water and methanol (3:1, v/v) was added, and the tubes were vortexed again and centrifuged at 5000 × g for 5 min at 10 °C. Following centrifugation, the upper phase was collected, evaporated, and stored at −20 °C until analysis.

The same LC–MS/MS equipment, column, and solvents as mentioned above were used for the determination of oxylipins. The solvent elution was carried out at a flow rate of 0.5 mL min^{−1} and was started with 70% A, decreased to 5% A over 2 min, and maintained at 95% B for 5 min before returning to the initial solvent composition over 2 min. The mass spectrometric analysis was carried out under the following conditions: spray voltage −4500 V, curtain gas 25 psi, nebulizer gas 50 psi, turbo gas 50 psi, and ion source temperature 500 °C. The quantitative analysis of oxylipins was performed using the multiple reaction monitoring (MRM) pairs (Bernat et al., 2018a).

2.7. Extracellular metabolites identification

The fungal cultures were filtered using a 115-mL filter unit (Thermo Scientific), and then 10 mL of the supernatant was transferred to a 50-

mL Falcon tube. The metabolites were extracted from the supernatant using a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) procedure (Paraszkiewicz et al., 2017). Briefly, the required salts (2 g MgSO_4 , 0.5 g NaCl , 0.5 g $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$, 0.25 g $\text{C}_6\text{H}_6\text{Na}_2\text{O}_7 \cdot 1.5\text{H}_2\text{O}$, and 10 mL acetonitrile) were added to the supernatant, and the samples were vortexed to completely dissolve them. Further, the samples were centrifuged at $4000 \times g$ for 10 min at 4°C . The upper phase was collected and stored at -20°C until analysis.

The same LC–MS/MS equipment as mentioned above was used for the determination of extracellular metabolites. Chromatographic separation was carried out using a Kinetex C18 column (50 mm \times 2.1 mm, particle size: 5 μm ; Phenomenex, USA; column temperature 40°C , injection volume 10 μL). The eluents used were water (A) and methanol (B) containing 5 mM ammonium formate. The solvent elution was performed at a constant flow rate of $500 \mu\text{L min}^{-1}$ and was started with 80% of eluent A for 0.25 min, and then decreased to 10% of eluent A, which was maintained for 4 min. Initial conditions were restored for the next 2 min. The MS/MS detection was performed in the positive-ionization MRM mode. The optimized parameters of the ESI ion source were as follows: CUR 25, IS 5500 V, temperature 500°C , GS1 50, and GS2 50. The monitored MRM pairs were m/z 366 > 320, 366 > 224 for harzianic acid and 345 > 259, 345 > 241 for t22-azaphilone.

2.8. Membrane permeability investigation

The permeability of the microbial membrane was determined in accordance with the method described in our previous paper (Stolarek et al., 2019). Briefly, 1 mL of the fungal culture was transferred to an Eppendorf tube and centrifuged for 5 min at $6000 \times g$. Then, 2 μL propidium iodide (PrI) solution (1 mg/mL in H_2O) was added to the mycelium sample suspended in 1 mL phosphate-buffered saline (PBS, pH 7) and incubated for 5 min at room temperature in the dark. Following incubation, the mycelium sample of *T. harzianum* was centrifuged for 5 min at $8000 \times g$ and the supernatant was removed. The collected pellet was washed three times with PBS. Then, the mycelium was suspended in 0.5 mL of PBS and transferred to a 24-well cell culture plate. The fluorescence intensity was measured in the plate using a FLUOstar Omega spectrofluorometer (BMG Labtech) with the following working parameters: λ_{ex} 540 nm and λ_{em} 630 nm.

2.9. Lipid peroxidation

Lipid peroxidation was determined in the samples according to the method of Gajewska et al. (2012), with some modifications. After completing the entire procedure, the samples were left for 10 min before they were used for spectrophotometric analysis.

2.9.1. H_2O_2 detection

Samples for H_2O_2 detection were prepared according to the method by Siewiera et al. (2017). 1 mL of the fungal culture was transferred to an Eppendorf tube and centrifuged for 2 min at $5000 \times g$. Then, 1 mg/mL DAB suspended in 1 mL phosphate-buffered saline (pH 3.8) was added and the samples were stained at room temperature for 30 min.

2.9.2. O_2^- detection

Samples were prepared for a Nitroblue tetrazolium (NBT) assay, according to the modified method by Siewiera et al. (2017). 1 mL of the fungal culture was transferred to an Eppendorf tube and centrifuged for 2 min at $5000 \times g$. Then, 0.1% NBT with 10 mM NaN_3 was added to the mycelium sample suspended in 1 mL phosphate-buffered saline (pH 7.8) and the samples were stained at room temperature in the dark for 15 min.

2.9.3. NO^\bullet detection

Samples were prepared according to the modified method by

Siewiera et al. (2017). 1 mL of the fungal culture was transferred to an Eppendorf tube and centrifuged for 2 min at $5000 \times g$. Then, 50 μM H_2DCFDA suspended in 1 mL phosphate-buffered saline was added to the mycelium sample and the samples were stained at room temperature in the dark for 10 min. The mycelium was rinsed 3 times with PBS buffer.

2.9.4. Peptaibol determination

Samples for the determination of peptaibol were prepared according to the same method as described for phospholipid isolation (Bernat et al., 2018b). Identification of peptaibols was carried out in agreement with papers censorious *Trichoderma* spp. peptaibols (Naher et al., 2014). The evaporated sample was diluted with 2% acetonitrile/0.1% trifluoroacetic acid. In the next step, 0.5 μL of the matrix (cyano-4-hydroxycinnamic acid matrix, solution 10 mg/mL) and 0.5 μL of the examined sample were applied on a 384 MALDI Opti-TOF 123 mm \times 81 mm plate. MALDI-TOF/TOF analyses were conducted in the positive ionization and reflector mode in the range m/z 900–2500 at a fixed laser intensity of 3500 (instrument-specific units). Then, the selected signals were chosen for the MS/MS analysis at a fixed laser intensity of 5000 (instrument-specific units).

2.9.5. Analysis of extracellular protein

The cultures were filtered with a 115-mL filter unit (Thermo Scientific), and then 10 mL of filtrate was aliquoted to five tubes (50 mL). Then, 40 mL of cooled acetone was added, and the mixture was left overnight (-20°C). The tubes were centrifuged at $8000 \times g$ for 10 min at 4°C . After decanting the supernatant, the precipitate was transferred to LoBind protein tubes (Eppendorf) and 500 μL of cooled acetone was added. Then, all the samples were homogenized using the FastPrep-24 homogenizer at a vibration frequency of 5 m s^{-1} for 30 s and centrifuged at $13,500 \times g$ for 5 min at 4°C . The purification step was repeated 3 times, and the last centrifugation step was extended by 10 min. The precipitate was dried under reduced pressure for 10 min at 30°C and dissolved in SB buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.01 M DTT) (Soboń et al., 2019). The amount of protein in the samples was determined using the Bradford method.

According to the method described by Szewczyk et al., 2014, the content of protein in the samples was analyzed initially by gel electrophoresis, with each sample diluted to an equal content of protein. The 6500- to 200,000-Da molecular mass marker (Sigma-Aldrich) was used as a gel calibrator. Protein digestion was performed using trypsin, and peptide sequences were determined using MALDI-TOF/TOF (Ab Sciex 5800 TOF/TOF system, USA) as described by Bernat et al. (2014). The Protein Pilot software v4.5 (Sciex) with a Mascot search engine v2.4 was used for protein identification. The obtained MS data were analyzed using NCBI nr database with taxonomy filter for *Trichoderma* (total number of sequences = 278,324).

To obtain the information on the function of proteins, especially for hypothetical proteins, the BLASTp algorithm in the nonredundant BLAST protein database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi> 2019) was performed.

2.9.6. Statistical analysis

Three replicates were prepared for testing. Standard deviations (SD) were determined in each sample. Statistical analyses were performed using a two-factorial and multifactorial analysis of variance with Tukey's post hoc test in the STATISTICA v.13.3 software. Statistically significant changes were accepted while $p < 0.05$.

3. Results

3.1. 2,4-D slightly inhibits fungal growth and it is not degraded by *T. harzianum*. In our preliminary studies we checked how different concentrations of 2,4-D (10–200 mg/L) influenced the fungal growth. The concentration of 100 mg/L did not inhibit *T. harzianum* growth, but it was noticed that the color of the culture medium changed, compared

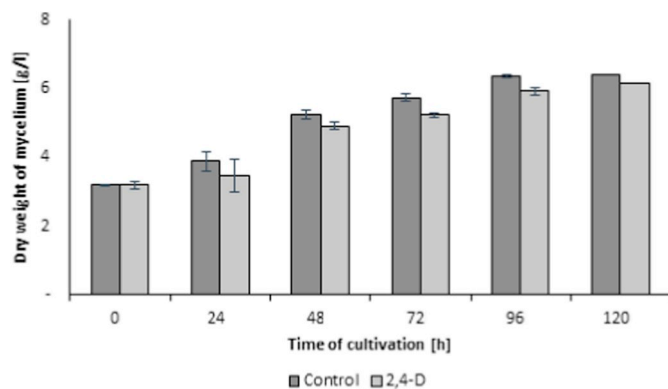


Fig. 1. Comparison of the dry mass of *T. harzianum* in the control culture and 2,4-D-exposed culture (100 mg/L) grown on Sabouraud medium.

to the control. Moreover, this concentration was used in other researchers' studies on 2,4-D degradation by soil fungi (Vroumsia et al., 2005; Itoh et al., 2013).

After exposure to 2,4-D (100 mg/L) for 5 days, the dry mass of mycelia was estimated. The inhibitory effect of the applied herbicide on the fungal growth rate was slightly visible (Fig. 1). After 5 days of incubation in Sabouraud medium, the dry mass of the control biomass and the biomass exposed to 2,4-D was 6.42 and 6.15 g/L, respectively. Using the LC-MS/MS technique, the amount of the herbicide was also measured in the fungal samples. However, no statistically significant changes in the amount of the added pesticide compared to abiotic control were observed (92 ± 3.7 , 96 ± 2.5 , for fungal sample and control, respectively) and no potential metabolites of its degradation were detected in the medium.

Taking the obtained results into account, all further experiments were conducted during the exponential (24 h) and the early stationary (96 h) growth phase of *T. harzianum*. No statistically significant changes in the herbicide treated culture, compared to the control, were detected.

During the fungal culture, pH measurements of the culture medium were taken. At the exponential growth phase the pH was 5.29 for the control medium and 4.81 for the medium with the herbicide, respectively. At the constant growth phase the pH for the control medium reached pH 4.09 while in 2,4-D presence it was 4.01.

3.2. Phosphatidylcholines and cardiolipin CL 72:8 decrease in *T. harzianum* mycelium exposed to the herbicide. Phospholipids belonging to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), and phosphatidylserine classes were determined using the LC-MS/MS technique. The quantitative analysis of the phospholipids of *T. harzianum* revealed that PC and PE classes dominated, constituting around 90% of the total composition of phospholipids in the fungi (Fig. 2). It was also found that the fungal biomass exposed to the herbicide had significantly higher levels of PE and lower levels of PC at both the examined growth phase; however, during the exponential growth phase, the difference in the levels of phospholipids was better visible. Moreover, the presence of 2,4-D induced an increase in the levels of PA and PI. The phospholipids of *T. harzianum* were found to be mainly composed of 16- and 18-carbon fatty acids, among which PC 18:2/18:2 and PE 16:0/18:2 species were dominating (Supplementary Fig. 1).

Another important class of phospholipids is cardiolipins. Three species of cardiolipins—CL 72:6, CL 72:7, and CL 72:8—were mainly identified from the examined fungal mycelium. The content of CL 72:8 species was found to be decreased in the presence of the herbicide, while an opposite trend was observed in the case of CL 72:7 (Fig. 3).

3.3. Ergosterol, sphingosine and dihydrosphingosine synthesis affected by 2,4-D. A decrease in the level of ergosterol was observed in the

samples with the addition of 2,4-D against the control. In the exponential phase of growth, the amounts of the sterol were 2.56 ± 0.17 and 1.87 ± 0.15 $\mu\text{g}/\text{mg}$ dry weight, for control and 2,4-D, respectively. Moreover, during the following days of incubation, the differences between the amounts of the sterol in both types of culture were also observed (4.6 ± 0.42 and 3.3 ± 0.14 $\mu\text{g}/\text{mg}$ dry weight, for control and 2,4-D, respectively).

T. harzianum produced various species of ceramides and dihydroceramides, which comprised about 70% of the detected sphingolipids. Other sphingolipids, such as sphingosine, sphingosine-1P, dihydrosphingosine and dihydrosphingosine-1P were also determined. The ceramide and dihydroceramides contents were found to be higher in 2,4-D-treated samples compared to the control while the level of dihydrosphingosine decreased (Supplementary Fig. 4).

3.4. Amounts of triacylglycerol with unsaturated fatty acids decrease in the presence of the herbicide. 18 species of TAG were identified (Supplementary Fig. 3). Among them, NH_4^+ adducts of TAGs of 54:6 and 52:4 predominated in *T. harzianum* (about 20% each) at the exponential phase of growth, followed by 52:3, 52:5 and 54:4 (about 10% each). Analysis of the distribution of the hydrocarbon chains in the exponential phase of growth showed that mycelium exposed to 2,4-D possessed a lower percentage of unsaturated species compared to the control sample (Supplementary Fig. 3).

3.5. 2,4-D induces lipid peroxidation in the mycelium. To identify if 2,4-D caused lipid peroxidation in the examined strain, the amounts of the peroxidation product were determined at both time periods. In each phase of growth, the amount of thiobarbituric acid-reactive substances (TBARS) was higher in the herbicide treated sample. At the exponential growth phase it was $1.20 \mu\text{M}/\text{mg}$ (± 0.03) in the control sample, while in the 2,4-D sample it reached $1.45 (\pm 0.1) \mu\text{M}/\text{mg}$. At the constant growth phase it was $1.13 \mu\text{M}/\text{mg}$ (± 0.03) in the control sample and $1.89 \mu\text{M}/\text{mg}$ (± 0.16) in the presence of 2,4-D. Also, the level of oxygenated fatty acids, termed oxylipins, was determined (Fig. 4), which showed significant differences in the content of 13-HODE and 9-HODE. In addition, statistically significant differences were observed in the content of 9-oxoODE, 13-oxoODE, and 13-HPODE. The level of the lipid 13-HODE, which was found to be dominating in the biomass exposed to 2,4-D, was twofold higher at both time intervals, compared to the control sample. On the other hand, the level of 9-HODE in the samples treated with the pesticide was decreased in the exponential and increased in the stationary growth phase. Interestingly, the amount of 13-HPODE was found to be drastically decreased in the fungal biomass harvested at the stationary growth phase.

3.6. 2,4-D exposure causes a release of reactive oxygen species (ROS). During the cultivation of *T. harzianum* with the 2,4D, there was observed a change in Redox balance. Using the NBT, H_2DCFDA and DAB assay the level of O_2^- , NO^\bullet , and H_2O_2 was measured and the percentage of a specific color of an image was analyzed (Supplementary Fig. 5). A higher amount of H_2O_2 8.66% (± 1.77) was observed in the herbicide treated sample, compared to the control 0.37% (± 0.26) (statistics H_2O_2 : $p = 0.0003$). Also, increased intracellular levels of superoxide anion in the culture with the herbicide 14.61% (± 5.57) and NO^\bullet 14.24% (± 4.7) during the exponential growth phase were noticed, in the control sample the presence of these ROS was not determined (statistics O_2^- : $p = 0.010$; NO^\bullet : $p = 0.0001$).

3.7. The herbicide inhibits the production of peptaibols, harzianic acid and t22-azaphilone, which possess antibiotic and plant growth-promoting activity. The effect of 2,4-D on the selected extracellular metabolites of *T. harzianum* was analyzed using the QuEChERS procedure. Two compounds—harzianic acid and t22-azaphilone—were identified in the culture filtrate. The mass spectra of these two compounds were recorded, and the obtained results were compared with the literature (Vinale et al., 2006, 2014). A significant difference in the level of these compounds was observed between the samples exposed to 2,4-D and the control samples (Fig. 5). During the exponential phase of growth, the amounts of harzianic acid and t22-azaphilone were fourfold lower

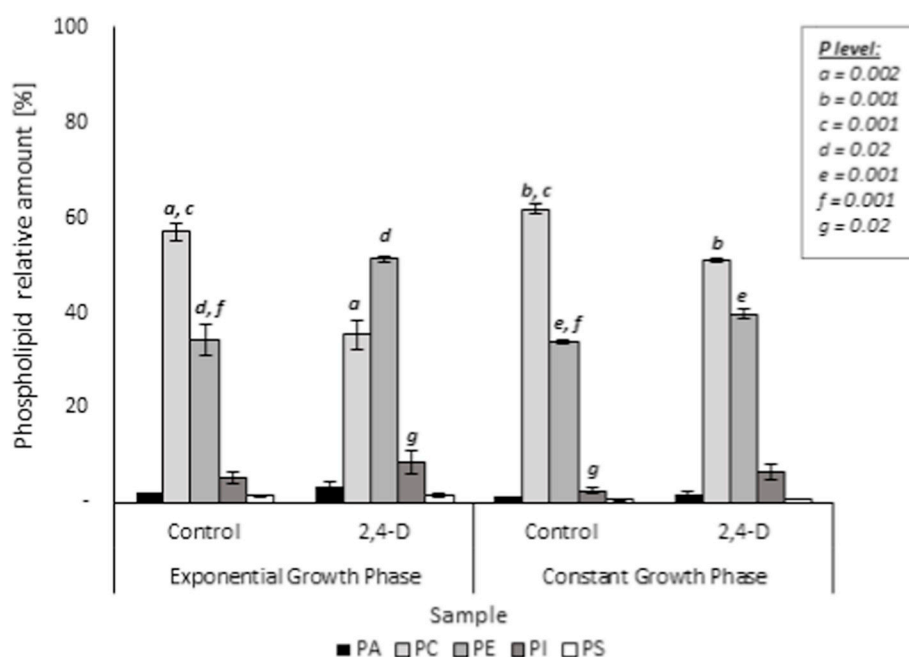


Fig. 2. Comparison of the content of phospholipids in the *T. harzianum* culture exposed to 2,4-D (100 mg/L) and in the control culture grown on Sabouraud medium. PA—phosphatidic acid, PC—phosphatidylcholine, PE—phosphatidylethanolamine, PS—phosphatidylserine, and PI—phosphatidylinositol.

in the herbicide-exposed fungal mycelium compared to the control sample. However, during the following days of incubation, the amount of harzianic acid was three times lower and that of t22-azaphilone was over 100 times higher in both types of culture.

To investigate the presence of peptaibols in the extract, MALDI-TOF/TOF spectra were collected. Samples analyzed in the reflector MS mode revealed the presence of many different ions, and the most prominent of these were around m/z 1380–1454. Analyzing the mass spectrum of the most dominant ion at m/z 1424.7 ($M + K$)⁺, a difference of 18 Da was observed, which was related to the loss of a water molecule (Supplementary Fig. 6). The results showed the presence of the short-sequence peptaibol (14 amino acids) with a partial sequence of amino acids, Ac-Aib-Gln, in the N-terminus.

3.8. 2,4-D influences the membrane permeability. After 1 and 4 days of incubation on Sabouraud medium, the fungal cultures were stained

with PrI, which is capable of passing through the damaged cell membrane and bind to DNA and can be detected using a fluorescence microscope. In the samples treated with the herbicide, an increase in the membrane permeability was observed on the control background at all the examined periods of growth (Fig. 6).

3.9. Analysis of extracellular proteomic response of *T. harzianum* to the herbicide. The analysis of the extracellular proteins of *T. harzianum* (using Bradford assay) revealed that the presence of the herbicide slightly influenced the protein excretion during the exponential phase of growth (1.2 ± 0.03 and 1.45 ± 0.15 mg/mL in control and 2,4-D-treated culture, respectively). However, during the following days of incubation, the differences observed between the amounts of proteins were significantly high in both types of culture ($p < 0.05$) (1.13 ± 0.03 and 1.89 ± 0.16 mg/mL in control and 2,4-D-treated culture, respectively). Furthermore, sodium dodecyl sulfate-

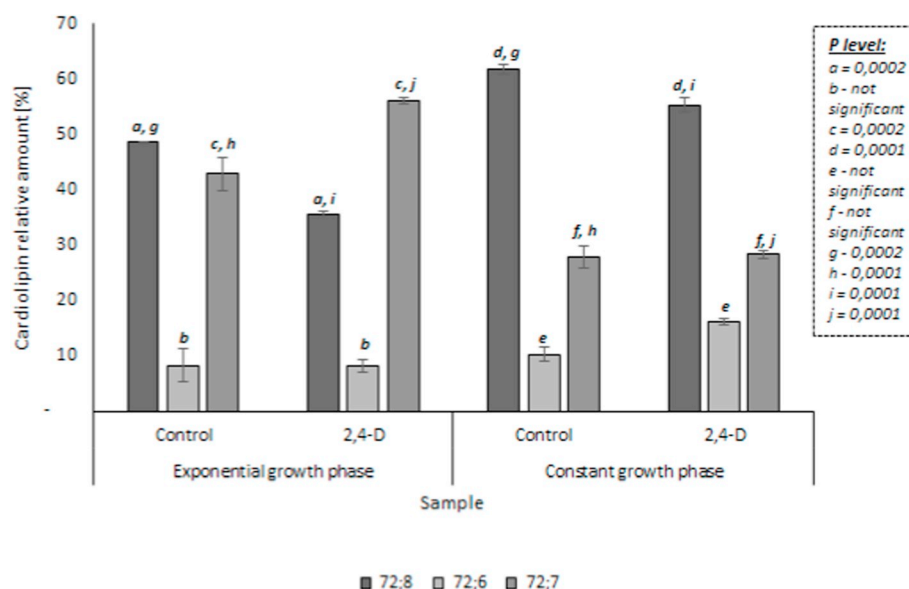


Fig. 3. Content of cardiolipin in the *T. harzianum* culture exposed to 2,4-D (100 mg/L) and in the control culture grown on Sabouraud medium.

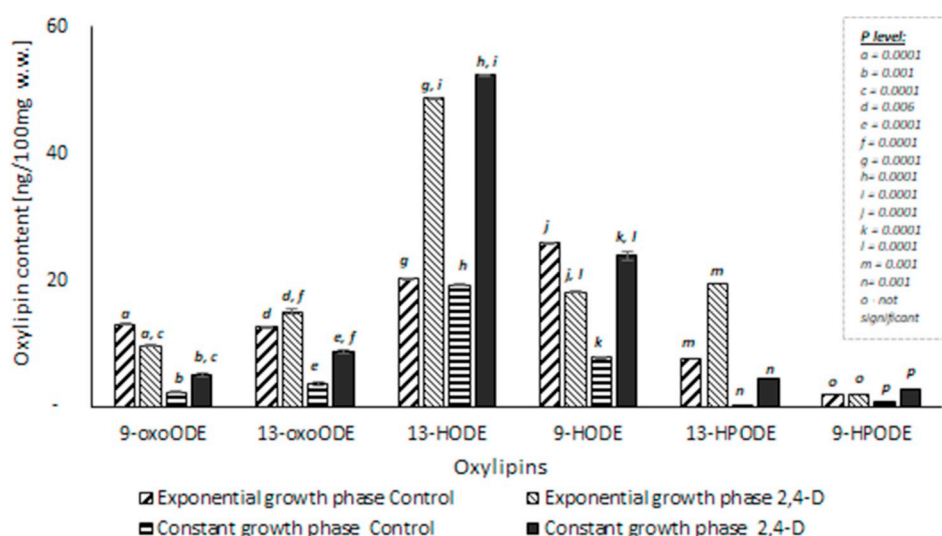


Fig. 4. Comparison of oxylin in the *T. harzianum* culture exposed to 2,4-D (100 mg/L) and in the control culture grown on Sabouraud medium.

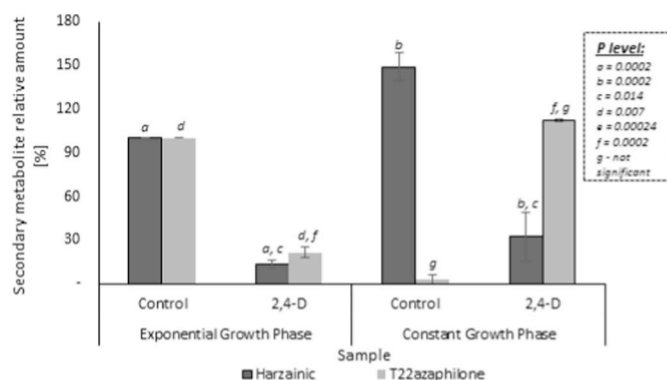


Fig. 5. Content of secondary metabolites in the *T. harzianum* culture exposed to 2,4-D (100 mg/L) and in the control culture grown on Sabouraud medium.

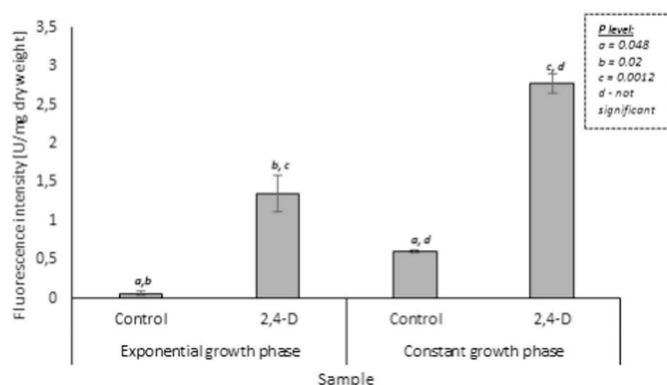


Fig. 6. Permeability of cell membrane in the *T. harzianum* culture exposed to 2,4-D (100 mg/L) and in the control culture grown on Sabouraud medium.

polyacrylamide gel electrophoresis revealed 17 major bands (Supplementary Fig. 2), which were found to be modified in the presence of the herbicide (Table 1). The obtained results demonstrated that phospholipase B (PLB)-like proteins and ceratoplatinin were found only in the herbicide-treated samples. On the other hand, phosphoesterase, FAD/FMN-containing dehydrogenase, transpeptidase, and acid phosphatase were identified only in the medium in which the control samples were grown.

4. Discussion

Due to their enormous capacity to produce secondary metabolites, *Trichoderma* is one of the most widely studied genera of fungi. This fungus has been applied as a biocontrol agent, as it can protect plants against pathogenic stress. However, it should be noted that the metabolic activity of *Trichoderma* in the soil can be affected by various biotic and abiotic stress factors, including the presence of pesticides. Among them, 2,4-D is commonly found in the environment and applied around the world to prevent the growth of weeds.

To determine the toxic effect of the herbicide on the fungal cells and to track the changes occurring at individual levels (proteome, lipidome, metabolome), necessary analyses were carried out. These include the determination of the defense mechanisms exhibited by the selected strain in the presence of the herbicide carried out by analyzing the synthesis of the extracellular compounds, as well as the changes in the development of the fungus.

The herbicide 2,4-D applied at a concentration of 100 mg/L to the *T. harzianum* culture showed mild inhibitory effect on the development of the fungus. Each of the growth phases was observed to begin with a slight delay in the 2,4-treated fungal culture compared to the control culture. It seemed that at the applied concentration, 2,4-D slightly negatively influenced the fungal growth to some extent. This finding was compared with another study which revealed that the strain IM 0961 was sensitive to metolachlor and resistant to alachlor at an initial chloroacetanilide concentration of 50 mg/L (Nykiel-Szymańska et al., 2019).

Auxins such as 2,4-D exhibit lipophilic properties and disturb the spatial organization of the fungal membranes and their functions (Viegas et al., 2005; Bernat et al., 2018b). Because pH of the culture medium was higher than the pKa of 2,4-D (pKa 2.73), the herbicide lost protons and was predominantly in the anionic form which did not adsorb more strongly to organic material than their neutral counterparts (Ramos de Andrade et al., 2014; Onthong et al., 2007). To verify how the herbicide affected the biological membranes of the fungal cells, the profile of phospholipids, which are the main component of the cell membrane, was investigated (Słaba et al., 2013). The results showed that PC and PE dominated among the phospholipids, confirming the results reported by other research works on *Trichoderma* (Gryz et al., 2019; Nykiel-Szymańska et al., 2019). Both the classes of phospholipids exhibit different properties: PC forms a bilayer structure, whereas PE makes the membrane more rigid (Bernat et al., 2014). Thus, the treatment of the fungal biomass with 2,4-D resulted in a slight decrease in the PC/PE ratio, possibly leading to a decrease in the membrane

Table 1
Effect of the presence of 2,4-D on the extracellular proteins of *T. harzianum*.

n.	Protein name	Function	Accession no.	Exponential growth phase		Constant growth phase		Score	MW (Da)
				Control	2,4-D	Control	2,4-D		
1.	Laminin G domain	Carbohydrate metabolic process [UniProtKB—A0A2K0U5M7]	PTB57655.1	+	+	+	+	139	72,973
2.	5'-Nucleotides	Hydrolase activity [UniProtKB—A0A1T3CKC5]	OPB41550.1	+	+	-	-	110	71,793
3.	Calcineurin-like phosphoesterase (MPPs); bifunctional 2',3'-cyclic nucleotide 2'-phosphodiesterase/3'-nucleotidase precursor protein	5'-Nucleotidase, calcineurin-like phosphoesterase, MPPs—have hydrolase activity [UniProtKB—A0A2T4A3L5]	KKP05350.1	+	-	+	-	441	71,849
4.	Fungal phospholipase B-like protein; lysophospholipase catalytic domain; cytoplasmic phospholipase A2; patatin	Phospholipase B hydrolyzes acyl groups resulting in storage of the lysophospholipid product (Kohler et al., 2006) Lysophospholipase removes the remaining acyl moiety on lysophospholipids (Channoum, 2000) Expression of patatin causes accumulation of palmitic acid and changes in the phospholipid profile. It is activated as a response to environmental stress or pathogen infection (Kohler et al., 2006) Serine binding directs a nucleophilic attack to hydrolyze covalent peptide bonds. It is involved in fungal development and pathogenesis processes (Pozo et al., 2004) Beta-lactamase related [UniProtKB—A0A2T4ABH4] Amidase—catalyzes hydrolysis of amide bonds (Kim et al., 2016) FAD binding [UniProtKB—A0A2T4A7H7] Transpeptidase superfamily—peptidoglycan glycosyltransferase activity [UniProtKB—A0A2N1LS61] Elongation in the cell wall of 1,3-beta-glucan linkage (glucan peptide chain formation by attaching molecules of 1,3-beta-glucan to nonreduced ones) [UniProtKB—A0A2T4A2I6] Bifunctional enzyme known to be involved in L-methionine biosynthesis [UniProtKB—A0A2T3ZTN5] Oxidoreductase activity [UniProtKB—A0A0F9XQ18] Berberine protein, sugar 1,4-lactone oxidases, FAD/FMN-containing dehydrogenase have oxidoreductase activity; main function is FAD binding [UniProtKB—A2BSM4] Aspartic-type endopeptidase activity [UniProtKB—A0A2N1LLV1] Hydrolyzes O-glycosyl compounds [UniProtKB—A0A0F9ZL66] Cerato-platanins (CPPs) present in the cell wall play a role in the growth and development of fungi (Bacelli, 2015). CPPs from pathogenic fungi are able to act as virulence factors in fungal-plant interactions. CPPs from fungi that are used as biocontrol agents, such as <i>Trichoderma</i> spp., cause a defensive reaction (Gardner et al., 2014) An elicitor of plant defense responses from <i>Trichoderma</i> (Viterbo et al., 2007)	PTB51325.1	-	+	-	+	572	70,002
5.	Serine protease; activation domain of S53 peptidases; peptidase domain in the S53 family; aorsin precursor		PKK54277.1	+	-	+	+	405	66,207
6.	Penicillin-binding transpeptidase domain		PTB54425.1	-	+	-	+	154	63,482
7.	Amidase superfamily		PTB50895.1	+	+	-	+	521	62,507
8.	FAD/FMN-containing dehydrogenase		PTB53020.1	-	-	+	-	456	61,929
9.	Transpeptidase superfamily; beta-lactamase; penicillin-binding protein transpeptidase domain		PKK52186.1	-	+	-	+	154	61,297
10.	Glycosyl hydrolase family; × 8 superfamily		PTB51281.1	+	+	+	+	486	57,119
11.	Acid phosphatase		PTB58363.1	-	-	+	-	86	54,041
12.	L-Galactono-1,4-lactone dehydrogenase		KKP02308.1	-	-	+	+	101	52,269
13.	Sugar 1,4-lactone oxidases; FAD/FMN-containing dehydrogenase; FAD-binding domain; berberine and berberine-like domain		PTB57084.1	+	+	+	+	306	42,302
14.	Aspartyl protease Endothiapepsin precursor		KKO97149.1	+	+	+	+	432	42,341
15.	Beta-1,3-endoglucanase		KKO98503.1	-	+	+	+	139	38,678
16.	Cerato-platanin		PTB54696.1	-	+	-	+	141	14,347
17.	Chain A, crystal structure of Sm1		pdb 3M3G A	+	+	-	+	203	12,538

fluidity.

In pea plants 2,4-D increased the activity of lipoxygenase (LOX), which can accelerate an oxidative degradation of fatty acids containing double bonds (Pazmiño et al., 2011). Therefore, it cannot be excluded that lipids peroxidation was responsible for the increased level of less unsaturated fatty acids observed in the mycelium. On the other hand, the herbicide could also inhibit the activity of Δ^{12} desaturase, which is responsible for the conversion of oleic acid (18:1 (n-9)) to linoleic acid (18:2 (n-6)). Moreover, the decreased level of PC and increased level of PE noted in the mycelium treated with 2,4-D could be associated with the inhibition of the methylation of PE with S-adenosylmethionine as a methyl donor to PC (Gooday, 1995). Other phospholipids such as PA, normally found in minor amounts in the cellular lipid pool, can increase significantly in plants in response to stress factors (Darwish et al., 2009). Also in the examined fungus the PA level increased in the presence of the herbicide. It cannot be excluded that phospholipase D catalyzes the breakdown of PC into choline and PA.

Cardiolipins are another class of phospholipids, but these are rarely studied in the filamentous fungi. In the present study, two species of cardiolipins were identified in the examined fungus: CL 72:8 (expected to contain four 18:2 fatty acyl groups) and CL 72:7 (contains three 18:2 and one 18:1 fatty acyl groups). Similar results were obtained in the studies on *Ustilago maydis* (Lambie et al., 2017). The changes in cardiolipins, with higher amounts of CL 72:7 and smaller levels of CL 72:8, observed in the study could have been associated with a decreased ratio of 18:2 versus 18:1 fatty acids in the herbicide-treated samples. Because cardiolipins form a component of the inner mitochondrial membrane, the above modifications could possibly influence the energy metabolism of the cells (Vähäheikkilä et al., 2018).

Triacylglycerols are a storage molecule of fatty acids in fungi (Pan et al., 2018). The obtained results for *T. harzianum* lipids revealed that in the presence of 2,4-D the TAG fraction was less enriched in polyunsaturated fatty acids. A similar observation had also been made in our previous studies for *U. isabellina* (Bernat et al., 2018b).

Sphingolipids are an integral component of fungal cell membranes (Heung et al., 2006). In *T. harzianum* cells, the level of ceramides was elevated in the herbicide presence. This is in agreement with some studies suggesting that ceramides are essential for mediating many stress responses. Wells et al. (1998) showed that sphingolipid-deficient strains of *S. cerevisiae* were unable to resist the heat shock. Moreover, the function of sterols and sphingolipids in eukaryotic cells is correlated (Gulati et al., 2010). Ergosterol is responsible for the membrane structure, function, and fluidity, and its proper level is crucial for the resistance to various stressful conditions (Suchodolski et al., 2019). In the present study, a decrease in the sterol was found in the mycelium of *T. harzianum* cultured with the herbicide compared to the control cells. Therefore, it cannot be excluded that the observed decrease in the level of ergosterol in the 2,4-D presence may have been compensated by the increase in the activity of ceramide synthase.

Modifications in the cell membrane could also be associated with its permeability (Risbo et al., 1997). The results of our previous study on *U. isabellina* (Bernat et al., 2018b) showed that 2,4-D increased membrane permeability and decreased membrane fluidity. Similarly, in the present study, the membrane permeability was found to be higher in the *Trichoderma* samples cultured in the presence of the herbicide.

The mechanism of the toxic action of 2,4-D toward weeds may be associated with the generation of ROS and lipid peroxidation. In the examined fungal mycelium treated with 2,4-D, the induction of oxidative stress was confirmed by the higher level of TBARS, a higher amount of H_2O_2 and the presence of O_2^- , $NO\cdot$, the amounts of which in the control were much lower or none. It is known that the response to oxidative stress is caused by disturbing the balance of the products and metabolites of ROS (Tudzyński et al., 2012).

Moreover, the elevated level of oxylipins and the hydroxyl derivatives of linoleic acid—9-HODE and 13-HODE—which are considered as the markers of lipid peroxidation (Spiteller and Spiteller, 1997)

confirmed that 2,4-D induced oxidative stress in *T. harzianum*.

Trichoderma fungi produce many extracellular metabolites, due to which these microorganisms affect the surrounding environment (Sivasithamparam and Ghisalberti, 1998; Ghisalberti and Sivasithamparam, 1991). Therefore, we examined how the presence of the herbicide influenced the synthesis of the selected extracellular compounds produced by the fungi. Using the LC-MS/MS technique, we determined the presence of harzianic acid and t22-azaphilone. Both compounds had been previously isolated from different *T. harzianum* strains (Vinale et al., 2006, 2008, Braun et al., 2018) and found to exhibit in vitro antibiotic activity against *R. solani*, *Pythium ultimum*, or *Sclerotinia sclerotiorum*. In addition, these compounds were shown to exhibit a growth-promoting effect on plants. In the present study, a decrease in their quantity was observed in the cultures treated with herbicide, and 2,4-D was found to negatively affect their biosynthesis. Another large family of compounds produced by *Trichoderma*—peptaibols—has also been found to exhibit antibiotic activity (Hermosa et al., 2014). They have a linear oligopeptide structure of 12–22 amino acids, are rich in alpha-aminoisobutyrate, and contain an acetyl group at the N-terminus and an amino alcohol group at the C-terminus (Naher et al., 2014). Using the MALDI-TOF/TOF technique, the 14-amino acid peptaibols were detected in the examined strain. The presence of similar compounds was reported by (Rebuffat et al. (1995)). The results of the present study showed that the peptaibol synthesis was inhibited by the herbicide in the exponential phase of growth, whereas in the stationary phase the inhibition was less visible.

In the proteomic analysis, the protein profiles of the control and pesticide-treated cultures were compared and some proteins were identified only in the 2,4-D-treated samples. Among them, PLB was found only in the herbicide-treated cultures at each time point. It has been reported that PLB proteins hydrolyze the acyl ester bonds in phospholipids and lysophospholipids and act as an important virulence factor in *Candida albicans* and other pathogenic fungi (Kohler et al., 2006). Another protein observed in the herbicide-treated culture was cerato-platanin, which acts as a natural plant elicitor (Bacelli, 2015).

During the proteome analysis, it was found that the herbicide inhibited the production of some proteins such as 5'-nucleotides, calcineurin-like phosphoesterase, and metallophosphatases. Cyclic nucleotide phosphodiesterase performs two functions—it acts as a messenger molecule in catabolic repression and modulates the properties of cell walls (Matange et al., 2015). In this study, these extracellular proteins were absent in the sample tested at the exponential growth phase. Laminin G modules, which play an important role in attaching the receptors to the cell in the extracellular environment, were present in both samples, which indicated that not all mechanisms were blocked by the herbicide.

Acid phosphatase is responsible for the mineralization of organic phosphorus. When the content of soluble phosphatase is low, the secretion of this enzyme is increased, and when its content is high, the secretion is suppressed. During the exponential phase of growth, acid phosphatases were found only in the control samples. Some researchers have highlighted that the addition of some chemicals could negatively affect the production of this enzyme (Nahas, 2015). For instance, a study revealed that the presence of Ni (II) ions in the medium in which *Rhizopus delemar* was cultured caused a decrease in the enzyme activity (Açikel and Ersan, 2010).

On the other hand, all the examined samples were found to contain aspartyl protease. This enzyme is involved in the activation of signal molecules and has been found to activate zymogens such as alkaline phosphatase in the fungi (Tacco et al., 2009). In addition, the protease produced by plants and microorganisms supports communication and signal expression (Li et al., 2016).

5. Conclusion

The present study demonstrated that 2,4-D significantly disturbs the

synthesis of lipids and extracellular metabolites in *T. harzianum*. Although this species has been widely studied, to the best of our knowledge, this study is the first to report the effect of 2,4-D on the activity of this fungus. The obtained data revealed that the presence of 2,4-D in the fungal culture induced oxidative stress including increased activity of LOX enzyme, disrupted the production of extracellular metabolites, and increased the membrane permeability of the fungal cells.

Thus, it cannot be excluded that ROS generation in fungal cells is correlated with increased activity of phospholipase D, which causes an elevated concentrations of PA and decreases PC. Also the herbicide presence could inhibit ergosterol synthesis and on the other hand activate ceramide synthase. However, for a proper understanding of the changes and enzymes activity occurring in fungal cells treated with 2,4-D more genetics and proteomics studies should be taken.

The presented data may be of significant value in the *partial* understanding of these processes and promote the application of *T. harzianum* in the soil in the presence of this pesticide.

CRediT authorship contribution statement

Julia Mironenka: Conceptualization, Investigation, Methodology, Writing - review & editing. **Sylwia Różalska:** Investigation, Methodology. **Adrian Soboń:** Investigation, Methodology. **Przemysław Bernat:** Investigation, Methodology, Funding acquisition, Writing - review & editing.

Declaration of competing interests

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

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Appendix A. Supplementary data

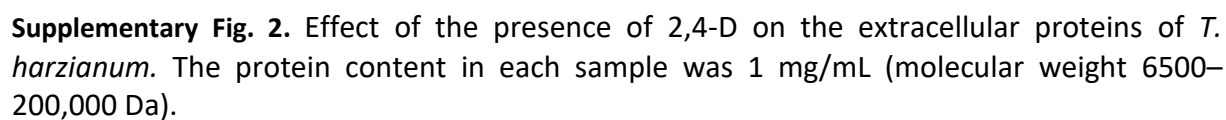
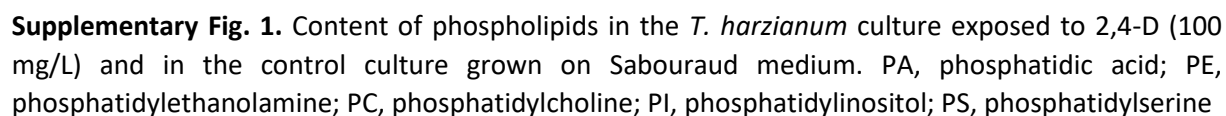
Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecoenv.2020.110383>.

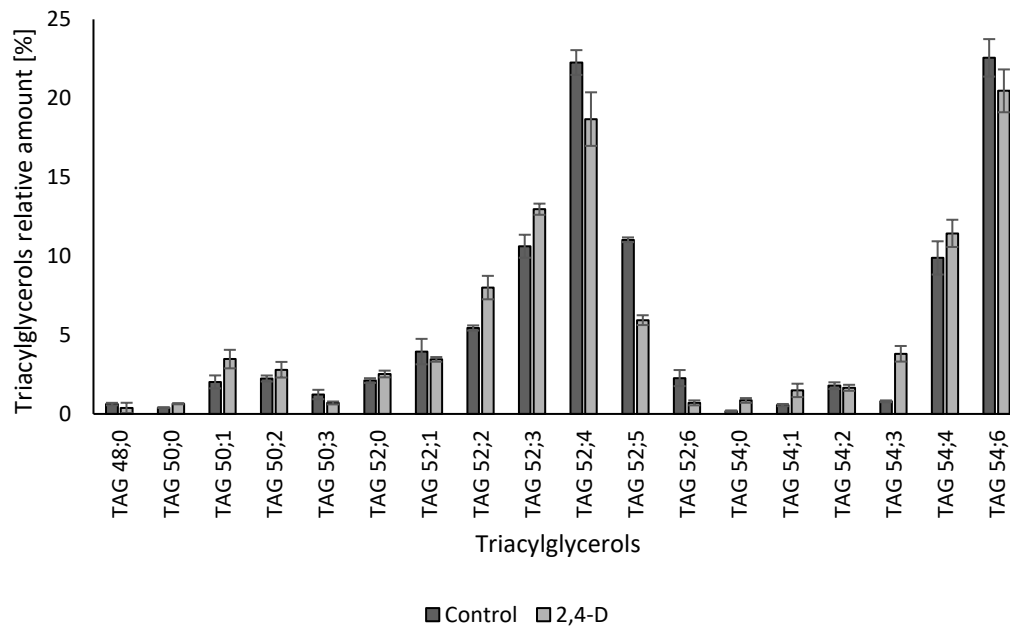
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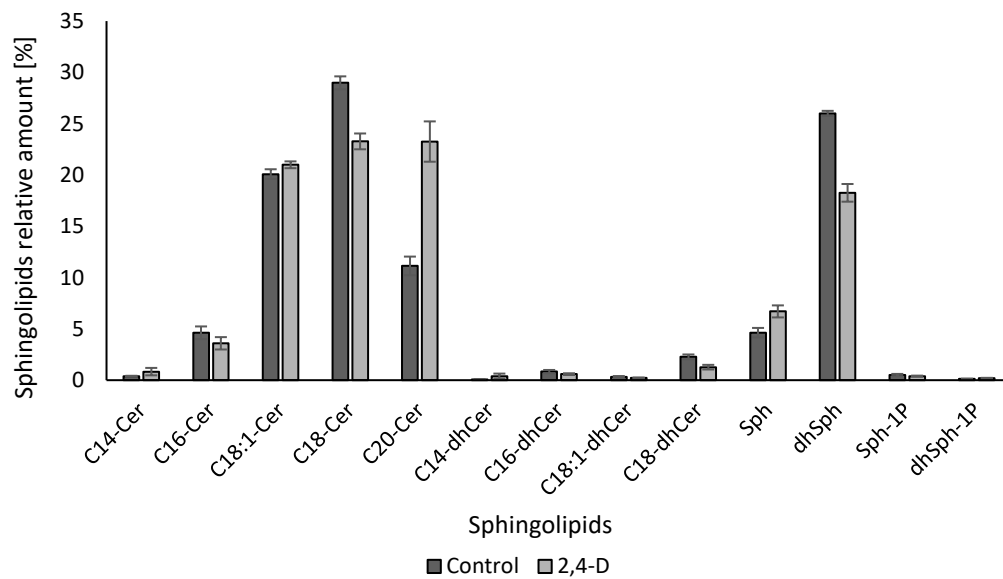
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P-1
Supplementary material

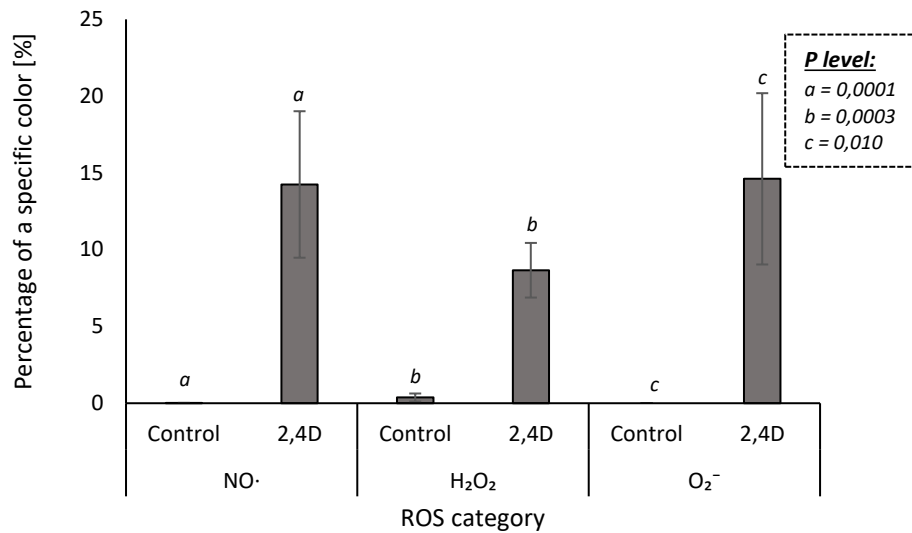




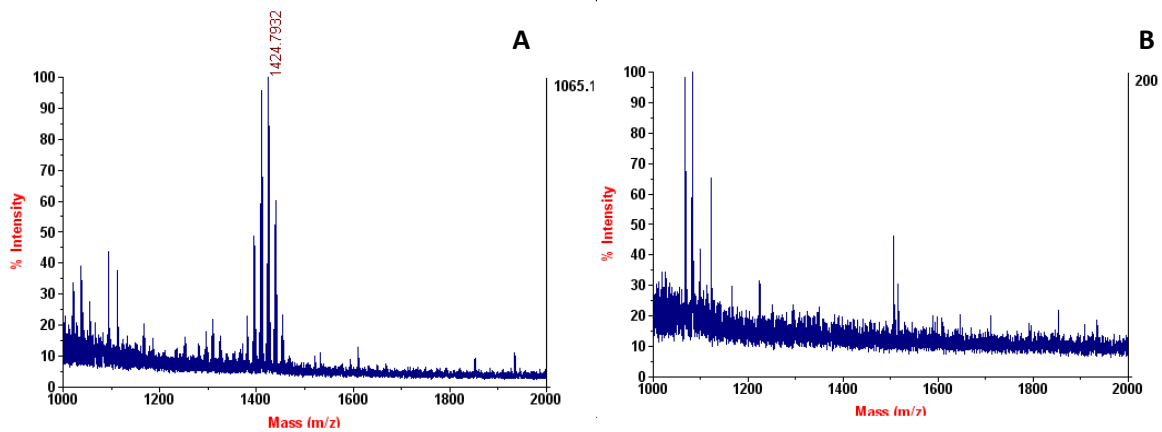
Supplementary Fig. 3. Triacylglycerols relative amounts in the *T. harzianum* culture exposed to 2,4-D (100 mg/L) and in the control culture at exponential growth phase, grown on Sabouraud medium. TAG, triacylglycerol.



Supplementary Fig. 4. Sphingolipids relative amounts in the *T. harzianum* culture exposed to 2,4-D (100 mg/L) and in the control culture at exponential growth phase, grown on Sabouraud medium. Cer, ceramide; dhCer, dihydroceramide; Sph, sphingosine; dhSph, dihydrosphingosine; Sph-1P, sphingosine-1-phosphate; dhSph-1P, dihydrosphingosine-1-phosphate



Supplementary Fig. 5. ROS levels in the *T. harzianum* culture exposed to 2,4-D (100 mg/L) and in the control culture at exponential growth phase, grown on Sabouraud medium.



Supplementary Fig. 6. MALDI-TOF spectra of peptaibols isolated from *T. harzianum* culture (A) and from the culture exposed to 2,4-D (100 mg/L) (B) at exponential growth phase, grown on Sabouraud medium.

W kolejnym etapie badań głównym celem było określenie wpływu metabolitów *T. harzianum* na patogen grzybowy *F. culmorum*. Doniesienia literaturowe wskazują, że wybrane szczepy *Trichoderma* mogą wykazywać działanie antagonistyczne wobec szczepów *Fusarium spp.* (np. działanie antagonistyczne wobec szczepu *F. oxysporum*, powodując hamowanie wzrostu grzybni wynoszące 75,7% i 67,7% w stosunku do kontroli) [14]. W niniejszej pracy do badań wykorzystano szczep *F. culmorum*, który w badaniach wstępnych miał największy wpływ na kiełkowanie pszenicy [dane nie publikowane], która stanowiła roślinny model badawczy w późniejszych etapach pracy doktorskiej.

Zewnątrzkomórkowe metabolity *T. harzianum* ekstrahowano z użyciem metody Quechers (ang. *quick, easy, cheap, effective, rugged, and safe*). Po przeprowadzeniu badań wstępnych, do dalszych doświadczeń wybrano ekstrakt z płynu pohodowlanego *T. harzianum* z 24 godziny hodowli, ponieważ zawierał najwyższe stężenie metabolitów takich jak kwas harzianowy, stanowiący główny związek odpowiedzialny za aktywność biologiczną (np. przeciwdrobnoustrojową, tworzenie antybiofilmu i dezagregację biofilmu) [15], czy peptaibole.

Badanie wzrostu w podłożu stałym z dodanymi metabolitami wymagało zaprojektowania odpowiedniej metody. W tym celu podłoże hodowlane ogrzewane w łaźni wodnej, po roztopieniu wzbogacano o równe ilości wcześniej przygotowanego ekstraktu z płynu pohodowlanego *T. harzianum* rozpuszczonego w etanolu lub wodzie, jako kontrolę podłoże wzbogacano o etanol lub wodę (porównanie rozpuszczalników dla odparowanych ekstraktów). Następnie podłoże z dodanym odpowiednim roztworem wylewano na szalkę Petriego, na którą po wystudzeniu nakładano zmyte ze skosów zarodniki *F. culmorum* (jedna kropla z ezy na środek szalki). Szalki Petriego inkubowano w 28°C przez 12 dni. Czas hodowli ustawiona eksperymentalnie, na podstawie wzrostu prób kontrolnych do osiągnięcia krawędzi szalki i zahamowania zmian we wzroście powierzchniowym.

Po inkubacji zmierzono i oceniono zmiany wywołane obecnością ekstraktów w podłożu stałym – odnotowano znaczne zahamowanie wzrostu *F. culmorum* na podłożach wzbogaczanych o ekstrakt z płynu pohodowlanego *T. harzianum*. Różnice dotyczyły także wykorzystanych rozpuszczalników po zatężaniu i odparowaniu ekstraktów. Próby z ekstraktem rozpuszczonym w wodzie, po 12 dniach

hodowli, choć nie osiągały rozmiarów hodowli kontrolnych, miały mniejszą różnicę w rozmiarach grzybni, natomiast próby wzbogacane o ekstrakt rozpuszczony w etanolu całkowicie hamowały wzrost badanego patogenu, różnica we wzroście wynosiła ponad 80% w porównaniu do kontroli (podłoże z etanolem). Po przeprowadzonym pomiarze masy grzybni, potwierdzono, że ekstrakt dodany do podłoża wpłynął na rozwój *F. culmorum* na podłożu stałym. Oprócz rozmiarów grzybni, zmiany dotyczyły także wyprodukowanych przez nie pigmentów – doniesienia wskazują, że zmiany zabarwienia mogą być wykorzystywane do analizy wzrostu grzybów z rodzaju *Fusarium* [16]. W przeprowadzonych badaniach zauważono zmianę w pigmentacji – próba kontrolna miała krwisto czerwone zabarwienie od 24 godziny hodowli, podczas gdy grzybnia traktowana ekstraktem przez cały okres hodowli pozostawała kremowo-białą.

Kolejny etap został zaplanowany do wykonania na hodowli płynnej *F. culmorum*. Jako rozpuszczalnik do ekstraktów zakwalifikowano etanol, ponieważ odnotowano wyraźne zmiany w rozwoju *F. culmorum*, w porównaniu do ekstraktu rozpuszczonego w wodzie i do kontroli.

Następny etap badań rozpoczęto od oszacowania wpływu dodanego ekstraktu z płynu pohodowlanego *T. harzianum* na wzrost *F. culmorum* w hodowli płynnej oraz odczyn pH podłoża pohodowlanego. Zaobserwowano, że wzrost grzybni został opóźniony a lag-faza uległa wydłużeniu w porównaniu do kontroli. Odczyn pH w tym czasie był niższy w próbie badanej pomiędzy 72 a 168 godziną prowadzonej hodowli. Zauważono również zmiany w wytwarzanych pigmentach, podobnie jak w hodowli na podłożu stałym.

Badania nad wytwarzanymi barwnikami rozpoczęto od oznaczania pigmentów naftochinonowych. W hodowli kontrolnej zaobserwowano dwie fale produkcji barwników - przed 96 i po 168 godzinie, gdy odczyn podłoża hodowlanego utrzymuje się na stałym poziomie. Próba kontrolna znacząco się różniła - największą ilość wytworzonych barwników odnotowano pomiędzy 120 a 168h. Zaobserwowano jednak pewne modyfikacje w produkcji dwóch głównych barwników – aurofusaryny i jej prekursora rubrofusaryny [17]. Począwszy od 144 godziny hodowli w obu próbkach oznaczono dużą ilość barwników, pH podłoża w tym czasie wynosiło powyżej 6, (według danych literaturowych stwarzało dogodne warunki

do ich produkcji) [18]. Nie zauważono wyraźnych zmian w produkcji aurofusaryny w badanych układach, natomiast odnotowano mniejsze stężenie rubrofusaryny w hodowli kontrolnej we wszystkich badanych punktach czasowych zaczynając od 120 godziny.

Zgodnie z nielicznymi danymi literaturowymi istnieje zależność pomiędzy pigmentacją a syntezą mykotoksyn przez *Fusarium* spp. [19] Także we wstępnych badaniach przeprowadzonych na tym etapie pracy doktorskiej, potwierdzono zdolność szczepu *F. culmorum* DSM1094 do produkcji mykotoksyn - zearalenonu.

Na podstawie dostępnych danych literaturowych można stwierdzić, że pewien czynnik hamujący syntezę toksycznego zearalenonu jest związany z syntezą aurofusaryny, ale sam mechanizm nie został jeszcze zdefiniowany [20]. Na tym etapie pracy doktorskiej odnotowano zmiany w produkcji barwników w hodowli płynnej i na podłożu stałym *F. culmorum*, zatem za pomocą celowanej metody LC-MS/MS postanowiono porównać ilość wyprodukowanych mykotoksyn w hodowlach rosnących na podłożach wzbogacanych o ekstrakt z płynu pohodowlanego *T. harzianum*. W hodowli kontrolnej na podłożu stałym stężenie zearalenonu wyniosło 27,16 ng/ml, w hodowli rosnącej na wzbogaconym podłożu stężenie było 10-krotnie niższe. Porównując zawartość hodowli płynnej, w 120h uzyskano wyniki: 25ng/ml dla układu kontrolnego i ok 2 ng/ml w hodowli rosnącej w podłożu z ekstraktem *T. harzianum*. Dodanie ekstraktu z płynu pohodowlanego *T. harzianum* okazało się być bardzo efektywnym inhibitorem syntezy mykotoksyn produkowanych przez badany szczep *Fusarium*. Mechanizm tego procesu, wciąż nie jest poznany, ale podjęto próbę oznaczenia zmian wywołanych obecnością dodanego ekstraktu na profil wewnątrzkomórkowego proteomu drobnoustroju z hodowli płynnej. W celu rozdzielenia wewnątrzkomórkowych białek *F. culmorum* zastosowano technikę dwukierunkowej elektroforezy (2DE – ang. *2-Dimensional Electrophoresis*), w której w oparciu o intensywność spotów na żelu przeprowadzono analizę porównawczą pomiędzy zidentyfikowanymi po strawieniu białkami, w hodowli kontrolnej i próbie badanej w 24h hodowli.

Białka, biorące udział w metabolizmie węglowodanów, przyczyniające się do szybkiego wzrostu grzybnii takie jak dehydrogenaza aldehyd-3-glicerynowa,

aldolaza, enolaza, spoty, były intensywniejsze na żelach pochodzących z układów kontrolnych.

W hodowli kontrolnej oznaczono hydrolazę S-formylglutationu, która jest odpowiedzialna za przekształcenie S-formylglutationu w glutation (GSH – ang. *glutathione*) i mrówczan [21, 22]. W próbce poddanej obróbce ekstraktem zauważono niezależną od GSH glioksalazę HSP31, która jest zaangażowana w reakcje ochronne przed RFT i katalizuje reakcję etyloglioksalu do D-mleczanu w pojedynczym, niezależnym od GSH etapie. Po zidentyfikowaniu powyższych białek postanowiono sprawdzić zawartość GSH w hodowlach.

GSH oznaczono w czterech punktach czasowych hodowli, które wytypowano na podstawie zmian odnotowanych we wcześniejszych badaniach, przeprowadzonych na tym etapie pracy doktorskiej (zmiany dotyczyły produkcji pigmentów, wzrostu oraz zmian pH). W hodowli kontrolnej zaobserwowano wzrost produkcji GSH, przypadający na 120 i 168h, natomiast hodowla traktowana ekstraktem z płynu pohodowlanego *T. harzianum* cechowała się mniejszym stężeniem GSH, pozostającym na równym poziomie przez cały okres hodowli. Doniesienia literaturowe wskazują, że deficyt GSH zwiększa ryzyko występowania stresu oksydacyjnego u grzybów [23]. Uzyskany wynik, a także zidentyfikowanie na żelu (2-D) enzymu katalazy (CAT – ang. *catalase*), której spot był intensywniejszy w hodowli badanej, może potwierdzać zachodzący stres oksydacyjny. Na podstawie otrzymanych wyników zbadano zawartości enzymów biorących udział w zwalczaniu reaktywnych form tlenu – dysmutazy ponadtlenkowej (SOD – ang. *superoxide dismutase*) i katalazy, za pomocą metody spektrofotometrycznej.

Mechanizm zwalczania reaktywnych form tlenu, zachodzi w dwóch falach - w pierwszej metaloenzymy takiej jak SOD dysmutują anion ponadtlenkowy, jako druga fala uaktywnia się katalaza, która detoksykuje nadtlenuk wodoru [24]. W wyniku przeprowadzonych badań w hodowli rosnącej na podłożu wzbogaconym o ekstrakt, zauważono wcześniej opisane dwie fale aktywności - aktywność pierwszej fali przypada pomiędzy 24-168h, drugie na 192h. W hodowli kontrolnej oznaczono niższy poziom aktywnej SOD, wyciszony po 168h w porównaniu do próby badanej oraz nieznaczny wzrost katalazy, który naturalnie występuje w hodowlach

grzybowych przy wykorzystaniu większości składników pokarmowych z podłoża hodowlanego.

Otrzymane na tym etapie badań wyniki wskazują, że dodanie do podłoża ekstraktów z płynu pohodowlanego *T. harzianum*, w którym między innymi zawarte są metabolity wtórne takie jak kwas harzianowy i T22-azofilon [dane niepublikowane], wpływają na wzrost *F. culmorum*, przyczyniając się do zachodzącego stresu oksydacyjnego, który zaburza normalne funkcjonowanie mikroorganizmu. Niewyjaśnionym nadal zostaje mechanizm wpływu na produkcję mykotoksyn i pigmentów poprzez badany grzyb z rodzaju *Fusarium*.

W przeprowadzonej części pracy doktorskiej zastosowano dwanaście metod badawczych, łącznie użyto 7 technik analitycznych do badań. Na podstawie otrzymanych wyników powstała autorska publikacja w czasopiśmie *Microbiological Research* (IF: 5,415; MNIŚW: 100), pod tytułem „*Trichoderma harzianum* metabolites disturb *Fusarium culmorum* metabolism: Metabolomic and proteomic studies”.

P-2

**“*Trichoderma harzianum metabolites disturb Fusarium culmorum metabolism:
Metabolomic and proteomic studies*”**

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Trichoderma harzianum metabolites disturb *Fusarium culmorum* metabolism: Metabolomic and proteomic studies

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ABSTRACT

Trichoderma species are well known for producing various secondary metabolites in response to different fungal pathogens. This paper reports the effects of the metabolites produced during one-day cultivation of *Trichoderma harzianum* on the growth and development of the popular pathogen *Fusarium culmorum*.

Inhibition of the growth of the pathogen and production of secondary metabolites including zearalenone was observed on Petri dishes. The presence of proteins such as cytochrome c oxidase subunit 4, glutathione-independent glyoxalase HSP31, and putative peroxiredoxin pmp20 in the extract-treated culture indicated oxidative stress, which was confirmed by the presence of a higher amount of catalase and dismutase in the later hours of the culture. A larger amount of enolase and glyceraldehyde 3-phosphate dehydrogenase resulted in faster growth, and the overexpression of stress protein and Woronin body major protein indicated the activation of defense mechanisms. In addition, a cardinal reduction in major mycotoxin production was noted.

1. Introduction

Fungi are well known producers of various metabolites, with useful biological activities (Boruta, 2017). Secondary metabolites are widely studied molecules with numerous applications in pharmacy and cosmetology, as food additives and as agrichemicals (Goyal et al., 2016). Also, secondary metabolites may play an antifungal role against agricultural phytopathogenic fungi (Khan et al., 2020). Currently, the possibilities of using these compounds for plant protection or as stimulants for plant growth have also been studied.

Trichoderma spp. are soil-borne fungi, which are known for their ability to adapt to external factors and survive in unfavorable conditions (Mukhopadhyay and Kumar, 2020). An advantage of these fungi is that they can produce a variety of enzymes such as amylase, glucanases and chitinases (Teixeira da Silva et al., 2017), metabolites, mainly terpenes, pyrones, gliotoxin, gliovirin, and peptaibols, which may express antifungal activity (Khan et al., 2020; Vinale et al., 2014). All of the compounds are involved in the communication and interaction with other organisms (Hermosa et al., 2014). *Trichoderma* spp. exhibit various antagonistic mechanisms against pathogens, such as the production of lytic enzymes, mycoparasitism, and competition for nutrients and space (Teixeira da Silva et al., 2017).

Trichoderma harzianum is widely studied owing to its plant protection capabilities. It produces secondary metabolites with antibiotic properties such as peptaibols (Vinale et al., 2014), harzianic acid (Vinale et al., 2009; Mironenka et al., 2020), trichoharzinan, and trichodermin (Li et al., 2019). The compounds produced by this species play an important role in tolerance to plant stress as well as in enhancing plant growth by increasing access to nutrients (Alwahibi et al., 2017). Also, secondary metabolites produced by *Trichoderma* may act as elicitors of plant defense mechanisms (Vinale et al., 2012). However, information about the effect of this species on phytopathogenic fungi is scarce.

Ferre and Santamarina (2010) described the role of chitinases, glucanases, and proteases produced by *T. harzianum* in the growth inhibition of *Fusarium culmorum*. Therefore, the current work, using modern techniques and "omics" research, may contribute to elucidating changes in pathogen physiology, such as development of the fungus and production of metabolites in response to *Trichoderma* metabolites.

The research was based on the possibility of using extracellular metabolites of *T. harzianum* to limit the growth and development of the phytopathogen *F. culmorum*. This species was selected as a fungal model because it is a popular cereal pathogen causing *Fusarium* head blight (FHB), foot and root rot (Kasprówicz et al., 2013). Together with *F. graminearum* it has been reported as predominant species of FHB

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disease (Kuzdraliński et al., 2014). *F. culmorum* has been isolated from sugar beet, flax, carnation, bean, pea, asparagus, red clover, hop, leeks, Norway spruce, strawberry and potato tuber (Scherm et al., 2012). The main mechanism behind the infection by this fungus is the production of mycotoxins — deoxynivalenol (DON), nivalenol, and zearalenone (ZEN), which pose a potential threat to human and animal health (Venkataramana et al., 2018).

Considering the above hypothesis, this study was designed with three objectives: (a) to compare the growth of *F. culmorum* on potato dextrose agar (PDA) plates treated with *T. harzianum* extract dissolved in water and ethanol (EtOH); (b) to perform a detailed analysis of *Fusarium* pigments and mycotoxin production; and (c) to monitor the effect of postculture medium extracts containing secondary metabolites produced by *T. harzianum* on the growth and development of *F. culmorum* on the metabolomic and proteomic levels.

2. Materials and methods

2.1. Reagents

Phospholipid standards were obtained from Avanti Polar Lipids (USA). Glutathione (GSH) and aurofusarin standards were obtained from Sigma Aldrich (Germany). All the chemicals used in the proteomic analysis were purchased from Bio-Rad, Promega (trypsin), and Sigma-Aldrich (6500–200,000-Da mass marker). The other materials, including solvents and plastics labware, were obtained from Avantor Performance Materials (Poland) and Eppendorf (Germany).

2.2. *Trichoderma* extracts

Spores isolated from 7-day cultures grown on ZT agar slants (containing (in g/L): glucose, 4; Difco yeast extract, 4; agar, 25; malt extract, 6° Balling [BLG] up to 1 L [1° BLG = 1 g of soluble substances extracted from the grain per 100 mL of malt extract]; pH 7.0) were used in the study.

The fungal spores inoculated in 20 mL Sabouraud dextrose broth medium (Difco) were added to 100 mL Erlenmeyer flasks (Bernat et al., 2018). The spores were cultured on a rotary shaker (160 ×g) for 48 h at 28 °C. Then, 2 mL of preculture was added to the Sabouraud medium. The cultures were incubated on a rotary shaker (160 ×g) at 28 °C. Following 24 h of incubation, the fungal cultures were filtered through a 115-mL filter unit (Thermo Scientific), and then 10 mL of the supernatant was transferred to a 50-mL Falcon tube.

The metabolites were extracted from the supernatant by the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method as it was described by Paraszewicz et al., 2017. The mix of salts (2 g MgSO₄, 0.5 g NaCl, 0.5 g C₆H₅NaO₇·2H₂O, 0.25 g C₆H₅NaO₇·1.5H₂O) and 10 mL acetonitrile were added to 10 mL of supernatant, and the samples were vortexed for 20 min. After dissolving the mix of salts, the samples were centrifuged 4000 ×g for 10 min at 4 °C. After extraction, 10 mL of the upper phase was collected and evaporated under pressure, and each extract was dissolved in 500 µL of EtOH or H₂O.

24 h extract was selected due to the high level of secondary metabolites measured in extracts, including the highest activity of beta-1,3-glucanase (important in pathogen cell wall destruction) noted at 24 h of liquid growth (Mironenka et al., 2020; Sánchez et al., 2007).

In the examined extracts, the presence of 14-aminoacids peptaibols as well as metabolites - T22-azophilone and harzianic acid – exhibiting antibiotic activity, was revealed (data not shown) (Naher et al., 2014; Mironenka et al., 2020).

2.3. Strain and growth conditions

The fungal strain *F. culmorum* DSM 1094 was purchased from the German Collection of Microorganisms and Cell Cultures GmbH. The fungal spores isolated from 7-day cultures grown on ZT agar slants were

inoculated as mentioned before. The preculture was added to the 20 mL of medium, containing previously prepared 10 mL of *T. harzianum* extract, evaporated under pressure and dissolved in 0.5 mL of ethanol. Controls were obtained by adding 0.5 mL of ethanol. The cultures were incubated on a rotary shaker under the aforementioned conditions. After 8 days of incubation, the fungal culture was exudate with a filter paper and quantified as described by Bernat et al. (2014). At the end of each day, the weight of dry biomass was measured using the method described by Nykiel-Szymańska et al. (2018). The liquid cultures were filtered using a vacuum pump, the mycelium which remained on the filter paper (Whatman no 2) was dried at 60 °C, until complete loss of moisture. Finally, the dry mass was weighed.

2.4. Growth on PDA plates

17 mL PDA agar slants were melted at 96 °C for 20 min in a laboratory water bath. Then, 500 µL of *Trichoderma* extract dissolved in EtOH or H₂O was added. Subsequently, the entire mixture was poured into 11-cm Petri dishes and cooled for 30 min. For the control sets 500 µL of EtOH or H₂O was applied. The lame lid was dipped in a saline-washed slant of *F. culmorum*, placed at the center of the Petri dish, and left for 20 min. Petri dishes were incubated at 28 °C for 12 days. At the end of each day, the size of the mycelium was measured.

The time of growth was experimentally selected, depending on the mycelial development, the growth of the mycelium was conducted until the colony reached the edge of the plate. After the control samples reached the maximum, the culture was carried out until the changes in the test samples had completely disappeared.

Following 12 days of incubation, the agar plates with mycelium were melted using boiled water, and the mycelium was transferred to a filter paper (Whatman no 2). Then, it was incubated for 2 h at 60 °C to attain dryness and weighed.

2.5. Spore quantification method

After incubation for 12 days, 10 mL of distilled water was added to the mycelium grown on Petri dishes. The entire content was smoothed out for 3 min using a laboratory coat, and the supernatant was collected into a 15-mL tube. The number of spores was counted using a Thoma chamber.

2.6. Mycotoxin extraction

100 mg of mycelium grown on a Petri dish was placed into a 50-mL tube, and 10 mL of acetonitrile:H₂O (75:25,v/v) was added. The samples were mixed on a shaker for 2 h and left overnight at 4 °C. On the following day, the tubes were centrifuged for 10 min, at 5000 ×g and 4 °C. The supernatant was filtered, and the mycotoxin content was determined by liquid chromatography with tandem mass spectrometry (LC–MS/MS).

The fungi grown in a liquid culture were transferred into 50-mL tubes with glass beads and homogenized for 3 min at the ball mill. Furthermore, 20 mL of ethyl acetate was added, and the tubes were placed on a shaker for 30 min. The tubes were centrifuged for 5 min, at 3500 ×g and 4 °C, and the upper phase was transferred to a new 50-mL tube; for the lower phase, a portion of ethyl octane was freshly added, and the entire procedure was repeated. The supernatant (40 mL) was filtered through filter papers and evaporated under pressure. Before the LC–MS/MS analysis, the extracts were resuspended in 1 mL of methanol.

The obtained mycotoxin extract was first fractionated using the Agilent 1200 HPLC system (USA). Chromatographic separation was performed using a Kinetex C18 column (50 mm × 2.1 mm, particle size: 5 µm; Phenomenex, USA; column temperature 40 °C, injection volume 10 µL). The eluents used were composed of water (A) and methanol (B), both containing 5 mM ammonium formate. The solvent was eluted at a constant flow rate of 500 µL min⁻¹, starting with 80 % of eluent A for

0.25 min, and then decreased to 10 % of eluent A, which was maintained for 4 min. The initial conditions were restored for further 2 min. The MS/MS detection was performed using multiple reaction monitoring (MRM) mode in the positive ionization. The optimized parameters of the electrospray ionization ion source were as follows: curtain gas (CUR) 25, ionization voltage (IS) 5500 V, temperature 500 °C, nebulizer gas (GS1) 50, and turbo gas (GS2) 50. The monitored multiple reaction monitoring (MRM) pairs were m/z 316.98 > 130.5 and 316.98 > 174.1 for zearalenone.

2.7. Phospholipid analysis

Phospholipids were extracted from biomass by applying the Folch method with slight modifications (Bernat et al., 2018). For the phospholipid analysis, 250 mg of wet biomass was transferred to a 2-mL Eppendorf tube containing glass beads. The samples were homogenized using the FastPrep-24 instrument (MP Biomedicals) at 5 m s^{-1} for 20 s (total time 1 min), then were vortexed for 3 min, and centrifuged for 10 min, at $5000 \times g$ and 4 °C. The supernatant was transferred to the new tubes, 200 μL of deionized water was added and the vortexing and centrifugation were repeated on the same parameters. The lower phase was collected, and stored at -70 °C.

The same LC-MS/MS equipment, column and solvents were used for phospholipid determination. The solvent was eluted at a flow rate of 0.5 mL/min, starting with 70 % A, then decreased to 5% A over 2 min, and maintained at 95 % B for 5 min before returning to the initial solvent composition for over 2 min. The mass spectrometric analysis was performed under the following conditions: spray voltage -4500 V, curtain gas 25 psi, nebulizer gas 50 psi, turbo gas 50 psi, and ion source temperature 500 °C. The phospholipids were quantitatively analyzed using the MRM pairs in negative mode (S1 Table) (Bernat et al., 2018).

2.8. Pigment identification and determination

The total amount of naphthoquinone pigments was measured using the method described by Medentsev et al. (2001), the absorbance was measured spectrophotometrically at $\lambda_{500\text{ nm}}$, and the value was converted by the coefficient of millimolar extinction 7.3.

Aurofusarin and rubrofusarin were extracted from the liquid cultures described by Westphal et al. (2018) with some modifications. Briefly, 10 mL of culture medium was transferred to 50-mL Falcon tubes. Then, 0.75 mL of HCl and 10 mL of chloroform were added, and the mixture was vortexed for 2 min. Subsequently, 7.5 mL of methanol was added, and the mixture was vortexed for 1 min. Next, the tubes were centrifuged at $5000 \times g$ for 5 min. The water phase was eliminated, 7.5 mL of 10 % NaCl with 3 mL of methanol was added, and the mixture was vortexed for 3 min. The organic phase was transferred to new Falcon tubes and evaporated under pressure for 2 h at 35 °C. Before the analysis, the extracts were diluted in 2 mL of chloroform:methanol mixture (1:1, v/v).

Flow injection analysis (FIA) was used to detect naphthoquinone pigments. The FIA-LC-MS/MS analyses were performed on an Agilent 1200 LC system coupled with a 4500 QTRAP mass spectrometer (Sciex). The mobile phase consisted of water:methanol (20:80) with the addition of 5 mmol/L ammonium formate. The eluent flow was 750 $\mu\text{L}/\text{min}$ for 1 min. The mass detector was set to the positive ionization MRM mode with an ESI (Turbo V) ion source. The optimized ion source parameters were as follows: CUR, 25; IS, 5500 V; temperature, 500 °C; GS1, 50; GS2, 50. The MRM pairs were m/z 571.2–556.3 and 273.1–230 for aurofusarin and rubrofusarin, respectively.

2.9. GSH analysis

GSH was analyzed using the modified method described by Slaba et al. (2015). Briefly, 250 mg of wet biomass was transferred to an Eppendorf tube, containing glass beads and 1 mL of cold H_2O . The samples were homogenized using the FastPrep-24 instrument at 5 m s^{-1}

for 20 s (total time 2 min, with a 2-min break on ice), centrifuged at $8000 \times g$ for 20 min at 4 °C, and 100 μL of the supernatant was transferred to another tube containing 900 μL of 1% formic acid (in EtOH). Following the incubation (2 h at -20 °C), the tubes were centrifuged at $12,000 \times g$ for 10 min at 4 °C. Then, the supernatant was transferred to new tubes and evaporated until dry at room temperature. The obtained extracts were resuspended in 1 mL of EtOH and analyzed by LC-MS/MS.

The FIA-LC-MS/MS analyses were performed according to the same system as above. The eluent consisted of water:methanol (50:50) with the addition of 5 mmol ammonium formate, at a flow rate of 750 $\mu\text{L}/\text{min}$. The detection of GSH was carried out using an MS/MS detector working in the negative ionization MRM mode. The monitored MRM pairs for GSH were m/z 306.1–143.1 and 306.1–272.1. The ion source and MS/MS parameters were as follows: CUR, 25; IS-4500 V; TEM, 550; GS1, 60; GS2, 50; collision gas (CAD), Medium; declustering potential (DP), -65; entrance potential (EP), -10; collision cell exit potential (CXP), -7.

2.10. SOD and CAT activity

The extract for enzymes activity measurement was performed according to the method by Moura et al. (2015). Briefly, 250 μg of wet biomass, was freeze-dried in liquid nitrogen in the mortar and 1 mL of extraction buffer (0.05 M sodium phosphate buffer pH 7.8, 1 mM EDTA, 1% PVP) was added. The samples were ground until a homogeneous mass was obtained. All was collected in the 15 mL tubes and centrifuged at $8000 \times g$ for 10 min at 4 °C. After centrifugation the supernatant was transferred to 1.5 mL Eppendorf tubes, and kept on ice during the analysis.

The catalase (CAT) and superoxide dismutase (SOD) activity was estimated by the method described by Nykiel-Szymańska et al. (2019). The CAT activity was determined by measuring H_2O_2 degradation at λ_{240} , while the SOD activity was measured by spectrophotometric evaluation of the inhibition of nitrotriazolium blue (NBT) chloride reduction at λ_{540} .

2.11. ROS measurement

H_2O_2 and O_2^- were measured by the method mentioned in our previous work (Mironenka et al., 2020). To identify O_2^- , the method of NBT reduction was used. The total amount of blue-stained radicals was determined under a microscope at magnification 40 \times . The level of H_2O_2 was measured by 3,3'-diaminobenzidine oxidation, and the amount of brown-colored radicals was determined under a microscope.

2.12. Intracellular protein extraction

The cultures were filtered through a 115-mL filter unit (Thermo Scientific), and the biomass was lyophilized in a vacuum dehydrator (Christ). Subsequently, 100 mg of biomass powder was added to 2 mL LoBind protein tubes (Eppendorf) containing glass beads. The samples were harvested and immediately homogenized using the FastPrep-24 homogenizer at a vibration frequency of 5 m s^{-1} for 30 s. The final step was repeated 3 times; followed by the final homogenization step, 1 mL of SB buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.01 M DTT) was added, and the samples were vortexed for 15 min. The tubes were centrifuged at $13,500 \times g$ for 10 min at 4 °C, and the supernatant was transferred to a new tube and centrifuged according to the same parameters. Following centrifugation, 300 μL of the supernatant was transferred to a new tube, 900 μL of cooled acetone was added, and the mixture was incubated for 1 h (-20 °C). After the supernatant was decanted, the precipitate was transferred to LoBind protein tubes (Eppendorf) and 500 μL of cooled acetone was added. Then, the purification step started - all the samples were homogenized using the FastPrep-24 homogenizer at a vibration frequency of 5 m s^{-1} for 30 s and centrifuged. The precipitate was washed with 90 % acetone, centrifuged

at 13,500 ×g for 5 min at 4 °C and next the supernatant was rejected. The last step was repeated 3 times, and the final centrifugation step was extended by 10 min. The obtained precipitate was dried under reduced pressure for 10 min at 30 °C and dissolved in SB buffer. The amount of protein in the samples was determined by the Bradford method.

2.13. 2-D SDS PAGE

Two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (2-D SDS PAGE) was performed by the modified method described by Sobon et al. (2019). For overnight passive rehydration, 300 µg of protein was added onto 11-cm IPG (immobilized pH gradient) strips of pH 3–10 NL (non-linear) (cat no.163–2016, Bio-Rad, Germany). Isoelectric focusing was performed using a Protean i12 device (Bio-Rad, Germany). The assay was prolonged according to the following parameters: 50 V for 4 h, linear gradient to 5500 V for 5 h, and held for 64,000 Vh. Equilibration and electrophoresis were carried out according to the modified manufacturer's protocol - the strips were incubated in 1% DTT equilibration buffer (50 mM Tris-Cl, 6 M urea, 30 % (v/v) glycerol, and 2% SDS) for 15 min, then strips were transferred into 4% IAA equilibration solution and placed on several polyacrylamide gels. A 6500–200,000-Da molecular mass marker (Sigma-Aldrich) was used as a gel calibrator.

The separation on the gels was repeated 3 times, to identify the differentiation, Image Master 2D Platinum 7 software (GE Healthcare, Germany) was used. A total of 124 spots from the control and 127 from the extract-treated sample were excised from the gels, digested and used for further analyses.

2.14. Protein identification

Protein digestion was performed by the modified method described by Szweczyk et al. (2014). Excised protein spots were digested with trypsin at 37 °C, overnight. Three different solutions were obtained for peptide extraction: 2% ACN with 0.2 % TFA (added for 20 min), 50 % ACN with 0.2 % TFA (added for 15 min), and 90 % ACN with 0.2 % TFA (added for 10 min) after each step, the supernatants were collected into one tube/sample. The received extracts were evaporated under pressure and dissolved in 50 % ACN 0.1 % TFA, imposed on an equal amount of α-cyano-4-hydroxycinnamic acid solution, and the peptide sequences were determined using matrix assisted laser desorption ionization-time of flight (MALDI-TOF)/TOF (Sciex 5800 TOF/TOF system, USA) as described by Bernat et al. (2014).

Protein Pilot software v4.5 (Sciex) with the Mascot search engine v2.4 was used for protein identification. The MS data were analyzed using the NCBI database with a taxonomy filter for *Hypocrea* (total number of sequences = 294,387).

To obtain the information on the function of hypothetical proteins, the BLASTp algorithm in the nonredundant BLAST protein database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi> 2019) was performed.

2.15. Statistical analysis

For all quantitative and metabolomic analyses, the experiment was carried out in three replicates, and the standard deviation was determined. Statistical analysis was performed using a two-factorial analysis of variance with the Tukey's post-hoc test in the STATISTICA v.13.3 software. The changes were significant at $p < 0.05$.

For proteomic analysis only those proteins identified by Mascot with a match Score greater than 72, which represented 5% confidence threshold, were considered statistically significant for analysis.

3. Results and discussion

Our previous study described the secondary metabolites produced by *T. harzianum*, which improve wheat germination, such as peptaibols,

harzianic acid, and T22-azofilone (Mironenka et al., 2020). Therefore, in the present work, we attempted to explain the effect of the extracellular extract of *T. harzianum* medium on the growth and development of *F. culmorum*.

3.1. Growth and development analysis of *F.culmorum* on PDA plates with *T.harzianum* extracts

To determine the effect of *T. harzianum* extract on *F. culmorum* growth, the sizes of the colonies on PDA plates were measured. For the test samples, controls were set up with the addition of an equal amount of solvents. The difference between the controls was not significant; however, the differences between the extract-treated samples (extracts included) and control were remarkable (Fig. 1).

The use of the mycelium color to analyze the growth prediction of *Fusarium graminearum* was investigated by Cambaza et al. (2018). The authors predicted the behavior of the fungus at various stages of its development based on its pigmentation. In our study, we noticed the color changes in the mycelium. The control sample was red from the first day of culturing and even after 12 days, while the extract-treated culture remained creamy yellowish-white, which could have been due to the lack of the precursor rubrofusarin (yellow) or transformation of aurofusarin (red) pigment.

For achieving clearer results, the size of the grown mycelium was measured every 48 h (Supplementary Fig. 1). A delay of the mycelium growth was observed on PDA plates, with extracts dissolved in both solvents, during the whole period of the study. The culture grown on PDA with extract dissolved in ethanol did not reach the control sizes and stopped growing after 12 days. The poor water solubility of some *Trichoderma* metabolites (i.e. peptaibols) (Zotti et al., 2020) resulted in smaller differences between the water extract and the ethanol extract.

To confirm the changes in the mycelium growth, an additional study was conducted to weigh the biomass. The mycelium treated with the extract dissolved in ethanol was found to be three times lighter than the control (Table 1), while the mycelium grown on the extract in water did not differ significantly in size.

To determine the impact on life processes, the number of spores was measured in each sample and the results obtained are presented in Table 1, indicating the effect of the extract on *F. culmorum* sporulation. Measurements showed a 5-times lower number of spores in the extract-treated samples compared to each control.

As a partial response to external stress, some secondary metabolites like pigments, microsporins, and some mycotoxins like fumonisins, thrichothecenes and zearalenone are produced (Perincherri et al., 2019); therefore, their content was analyzed after 12 days of culture. A lower ZEN content was found in the samples with the extract dissolved in ethanol, while no significant changes in the ZEN content were noticed between the control and the aqueous extract. Due to major changes – inhibiting the growth, sporulation and production of ZEN, the extract dissolved in ethanol was selected for further study.

3.2. Growth and development analysis of *F.culmorum* in flasks breeding with *T.harzianum* extracts

3.2.1. Growth condition and pH of culture medium

The inhibitory effect caused by the exposure of *T. harzianum* extract on the liquid culture of *F. culmorum* was significant at the first four time points (Fig. 2). However, after 120 h of culturing, a statistically significant better growth was noted in the extract-treated culture. It seems that in the presence of the extract, the lag phase of *F. culmorum* growth was prolonged, by at least 24 h.

The pH of the culture medium, Fig. 2, changed significantly from 7.2 to 16.8 h, which might have been related to the disruption of extracellular metabolites production. It is known that pH and oxidative stress affect the production of metabolites such as dyes and mycotoxins (Marín et al., 2010; Parincherri et al., 2019).

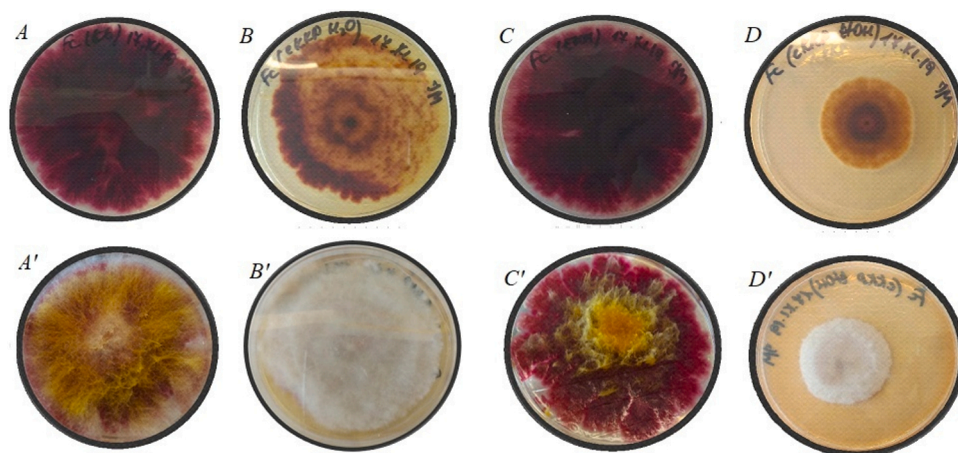


Fig. 1. 12-day *F. culmorum* colonies on PDA plates after 12 days of cultivation, 28 °C: The control samples (A, C), reached the maximum possible sizes, the mycelium was burgundy-red in color, with orange-yellow surface growth (A', C'). The colony grown on PDA with extract dissolved in water (B) was similar in size to the control, the color of mycelium - yellow cream with red places, with white surface (B'). The sample with extract dissolved in ethanol (D) did not grow to the level similar to control, the color of mycelium was red inside and yellow on the edges, with white surface (D'). See also Table 1.

Table 1

Comparison of the weight of mycelium and spore-producing *Fusarium culmorum* on Petri dishes, treated with *Trichoderma harzianum* extract on the 12th day of culturing. Data represent mean \pm SE. * are significantly different from control ($p < 0.05$).

	Control (H ₂ O)	Extract treated (H ₂ O)	Control (EtOH)	Extract treated (EtOH)
Weight of mycelium [g]	0.87 \pm 0.025	0.66 \pm 0.01*	0.84 \pm 0.01	0.13 \pm 0.02*
Spores/biomass [U/g]	91.38 \pm 3.94	8.16 \pm 1.22*	76.12 \pm 4.74	0.812 \pm 0.3*
Zearalenone concentration [ng/mL]	7.36 \pm 0.64	5.99 \pm 0.19	27.16 \pm 1.14	2.89 \pm 0.23*

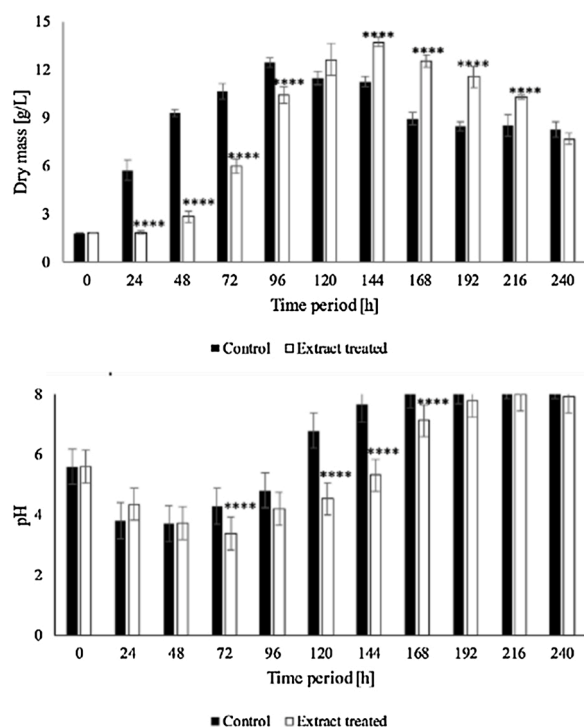


Fig. 2. Comparison of growth conditions and pH level of *F. culmorum* liquid culture treated with *T. harzianum* extract and the control (EtOH). Data represent mean \pm SE. **** are significantly different from control at each time period ($p \leq 0.001$).

3.2.2. Changes in extracellular pigment production

During the qualitative analysis of the *Fusarium* culture filtrate by the LC–MS/MS system, two pigments—aurofusarin and rubrofusarin—were identified. During the experiment, changes in the production of naphthoquinone pigments were observed, and the total amount of this group of dyes was measured (Fig. 3).

During the 10-day culture, the production of pigments in the control occurred in two waves (Fig. 3): one at the exponential phase of growth and the other accompanying the production of mycotoxins.

At the initial phase of growth, inhibition of dye production was noticed in the extract-treated sample, which may be correlated with the delay of mycelium growth, as shown previously (Fig. 2). A decrease in the total amount of naphthoquinone pigments was detected at 120 h in the control sample, while an increase in the amount of these dyes was noted in the extract-treated sample. However, some modifications were observed in the production of the two main dyes—significant changes in the amount of aurofusarin (↓) and its precursor rubrofusarin (↑) in the control compared to earlier measurements. Starting at 144 h, a high amount of dyes was produced in both samples, and the pH of the substrate was above 6 (Fig. 2) which additionally created favorable conditions for their production (Wu et al., 2017). The changes in aurofusarin production were statistically insignificant, and the values were comparable, in contrast to the lower rubrofusarin production observed in the control culture at all the other time points.

It is known that the production of dyes and mycotoxins is promoted at pH > 6 (Medentsev et al., 2005; Wu et al., 2017). Moreover, it has been found that pH 8 is optimal for the synthesis of the dimeric naphthoquinone—aurofusarin, and lower pH is favorable for the generation of the precursor of this compound—rubrofusarin—in *F. graminearum* (Malz et al., 2005). Spectrophotometric measurements of the total concentration of naphthoquinone dyes indicated a considerable dispersion of the produced dyes in the extract-treated samples. In the control, two waves of maximum dye production –were observed—the first one before 96 h and the other after 168 h when the pH became stable and the fungus growth stopped as described by Medentsev et al. (2005). The extract-treated sample had a completely different effect in this respect; the largest amount of dyes produced was marked between 120 and 168 h, and the polymerization of the fungal pigments may be associated with the formation of the oxidative compound (Rao et al., 2017).

During the experiments, the most important changes in growth included the highest increase in the fungal biomass in the extract treated culture at 120 h and the delay of biomass decline at 168 h. These time points were selected for the following analyses. Also, the total amount of naphthoquinone pigments was statistically changed in these time periods. In addition, 24 and 240 h of culturing were selected as the first and last time point for comparing the changes.

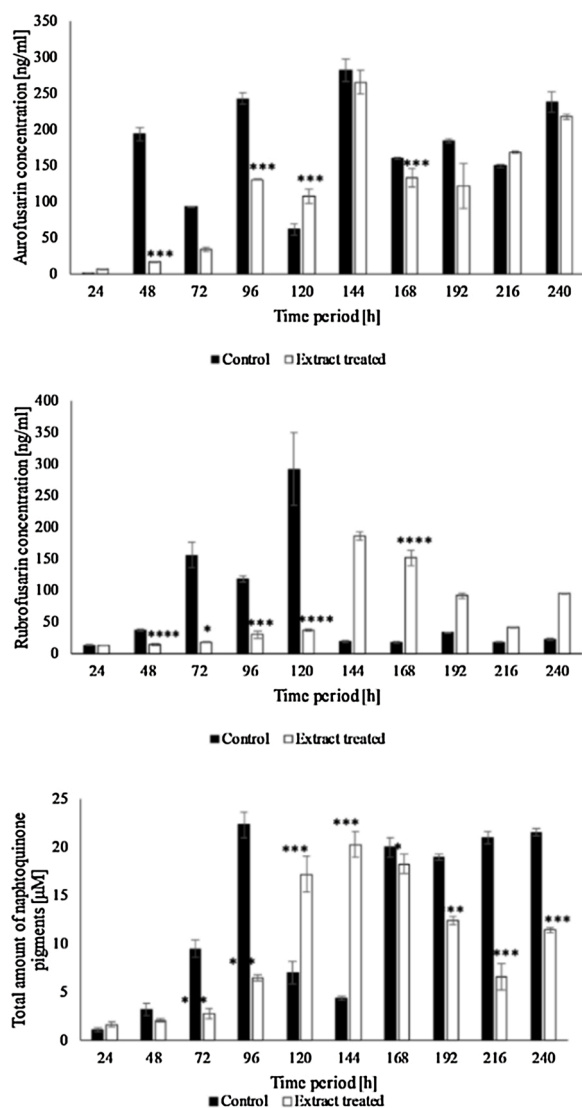


Fig. 3. Comparison of pigments produced on the liquid culture of *F. culmorum* treated with *T. harzianum* extracts (bars—aurofusarin and rubrofusarin total content, curve—total amount of naphthoquinone pigments). Data represent mean \pm SE. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.2.3. Production of mycotoxin

To investigate the effects of the *T. harzianum* extracellular metabolite on mycotoxin production, the amount of ZEN produced in the culture treated with the extract was examined. *F. culmorum* treated with the extract did not produce the amounts of ZEN comparable to the control culture (Fig. 4) during the entire breeding period. The highest concentration (26.65 ng/mL) in the control culture was detected at 120 h, when the pH was closest to 7. The mycotoxin was the most stable in the medium (Ryu et al., 2003), and subsequently reached the final concentration of 15.8 ng/mL.

Neuhof et al. (2008), who studied the distribution of ZEN in wheat kernels, found that a higher level of ZEN was observed in a group of reddish kernels (containing aurofusarin). However, during the determination of ZEN in the present study, the reddish extract did not reflect the amount of mycotoxin. Moreover, in the control sample, the maximum amount of analyzed mycotoxin was observed when the level of the red dye decreased; however, the concentration of rubrofusarin reached its maximum. The subsequent accumulation of ZEN was not dependent on the production of dyes. During the entire cultivation period, the amount of mycotoxin in the extract-treated sample was very low, probably due to the activation of defense mechanisms.

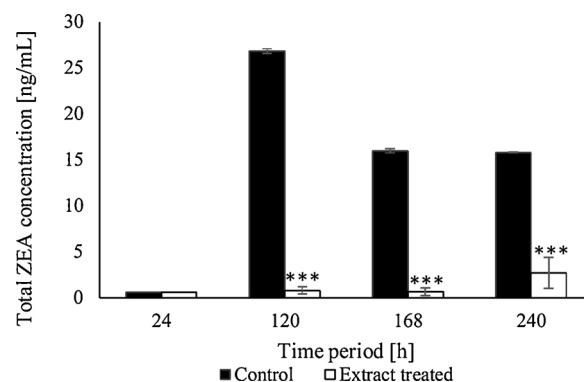


Fig. 4. Comparison of ZEN production by *F. culmorum* treated with *T. harzianum* extracts. Data represent mean \pm SE. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

During the study on a dual-plate culture of *F. graminearum* and different types of *Trichoderma*, Tian et al. (2018) observed that the inhibition rate of ZEN production ranged from 9 to 97 %, due to the antagonistic activity of *Trichoderma*. Our experiments on Petri dishes and flasks proved that the extracts containing metabolites exhibited antagonistic properties.

3.2.4. Changes in intracellular proteome

To determine the stimuli that induced the changes in the *F. culmorum* culture, treated with *T. harzianum* extract, the intracellular proteomics was assayed. Following triple2-D SDS-PAGE analysis, a total of 265 matched spots from both samples were excised from the gels (Supplementary Fig. 2), digested, and analyzed using MALDI-TOF/TOF followed by protein identification performed by the ProteinPilot software. All significant differences in the total spot volume, and Mascot Scores above 72 (5% of confidence threshold) are presented in Table 2.

Comparing the protein profile, the proteins required for carbohydrate transport and metabolism, such as glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase and enolase, described in the work of Li et al. (2015), playing a major role in the rapid growth and high pathogenicity of the fungus, were detected in higher amounts in the control culture.

Electron transport chains are essential mechanisms of chemical energy production, which support the chemical redox reaction. In these mechanisms, cytochrome c oxidoreductase (cytochrome bc1 complex) plays an important role, being the central component of the respiratory electron transport chain (Smith et al., 2012). During the proteomic study, a higher spot volume of the cytochrome b-c1 complex subunit (4.06), and the cytochrome c oxidase subunit (16.32) was found in the control culture, compared to the culture treated with the extract (1.06 and 3.26 respectively).

It was noticed that in the control, the expression of disulfide isomerase—responsible for protein folding and transport of electrons from GSH to NADPH (Gruber et al., 2006)—was higher, while in the extract-treated sample, the 78-kDa glucose-regulated protein was expressed, the synthesis of which was accompanied by excessive accumulation of unfolded polypeptides (Tiware et al., 2015). This protein is responsible for the formation of multimeric protein complexes inside the ER (Schroder and Kaufman, 2005). In the control culture, S-formylglutathione hydrolase was marked, which is a glutathione thiol esterase that hydrolyzes S-formylglutathione to GSH and formate (Papavasileiou et al., 2020; Wojtasik et al., 2011). In the extract-treated sample, GSH-independent glyoxalase HSP31 was noticed, which is involved in the protection against ROS and catalyzes the reaction of ethylglyoxal (MG) to D-lactate in a single GSH-independent step (<http://www.uniprot.org/uniprot/Q04432> accession 16.10.20).

Among the proteins present in the extract-treated sample, the attention is paid to translationally controlled tumor protein from

Table 2Differences in the set of intracellular proteins in the control and the extract-treated *F. culmorum* culture after 24 h of incubation.

Spot nr.	Protein ID*	Mass (kDa)	Proteins	Function	MascotScore**	Spot volume [x10 ⁺⁴]***	
						Control	Extracttreated
1	PTD11127.1	25	Cytochrome c oxidase subunit 4, mitochondrial	Participate in electrons transfer from NADH, creating an electrochemical gradient across the inner membrane, directs transmembrane transport and ATP synthase (Hartley et al., 2019)	293	16.32	3.26
2	PTD11704.1	46	Cytochrome b-c1 complex subunit 2, mitochondrial		235	4.063	1.06
3	PTD12993.1	20	ATP synthase subunit 5, mitochondria	Produces ATP from ADP in the presence of a proton gradient (Ghaemmaghami et al., 2003)	451	15.55	3.059
4	ESU14732.1	25	vacuolar ATP synthase subunit E		120	5.508	3.84
5	PTD07392.1	57	ATP synthase subunit beta, mitochondrial		934	34.9	12.6
6	PTD09210.1	18	Tropomyosin-2	Stabilizes actin filaments (Drees et al., 1995)	175	23.77	16.18
7	PTD10421.1	16	Actin	Structural constituent, involved in cytokinesis (Berepiki et al., 2011).	152	16.76	46.71
8	PTD12804.1	48	Enolase	Carbohydrate degradation. Participate in glycolysis pathway (Ghaemmaghami et al., 2003)	553	20.73	6.97
9	PCD19150.1	39	Glyceraldehyde-3-phosphate dehydrogenase	Catalyzes the conversion of DHAB to fructose 1,6-bisphosphate (Zgiby et al., 2000)	386	22.11	5.85
10	PTD11183.1	39	Fructose-bisphosphate aldolase	Catalyzes the conversion of unbranched alcohols to the corresponding aldehydes (Dhokan et al., 2016)	420	4.51	1.86
11	PCD36852.1	37	Alcohol dehydrogenase		190	1.76	8.6
12	PTD10299.1	16	Agaricusbisporus lectin	Binds disaccharide. (Yu et al., 1999).	326	4.3	1.95
13	PTD12831.1	53	Aldehyde dehydrogenase	Participate in biosynthesis of electron transport chain component (saint-Prix et al., 2004)	285	7.35	14.2
14	PTD12314.1	35	Protein GVP36	Endocytosis, vacuole organization (Ghaemmaghami et al., 2003)	142	9.99	4.43
15	PTD11204.1	55	Disulfide isomerase	Oxidative folding of proteins (Gruber et al., 2006)	809	2.93	0.7
16	PTD09879.1	31	S-formylglutathione hydrolase	Glutathione thiol esterase, hydrolases S-formylglutathione to GSH and formate (Papavasileiou et al., 2020)	400	3.25	14.67
17	PTD08746.1	42	Vacuolar protease A	Required for mitosis, meiosis, spore formation (Salamini et al., 2010)	427	1.58	5.46
18	PTD05987.1	16	Nascent polypeptide-associated complex subunit beta	Transcription activator (Rospert et al., 2002)	82	6.21	31.92
19	PTD09273.1	45	Putative nucleosome assembly protein		123	1.34	2.54
20	PTD09712.1	85	Aconitate hydratase, mitochondrial	Participate in the citric acid cycle (Fazius et al., 2012)	172	1.31	7.41
21	PTD04810.1	37	Transaldolase	Participate in pentose-phosphate pathway (Kourtoglou et al., 2008)	261	5.73	21.4
22	PTD04913.1	72	Heat shock 70 kDa protein	Stress response, folding of nascent polypeptide (Tiwari et al., 2015)	501	2.53	11.85
23	PTD01502.1	72	78 kDa glucose-regulated protein		316	3.34	7.82
24	PTD10541.1	28	Stress protein		1180	422	505
25	PTD13318.1	26	Minor allergen Alt a 7	Signaling proteins, small allergen molecules (Bakar et al., 2020)	607	11.31	31.42
26	PTD05982.1	61	Putative serine carboxypeptidase		147	85.97	35.46
27	PTD09103.1	85	Putative 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	Methionine formation, participate in virulence (Sun et al., 2014)	111	2.44	5.15
28	PTD09824.1	75	Alcohol oxidase	Oxidation of methanol to formaldehyde and hydrogen peroxide (M.A. Kwon et al., 2009; S.J. Kwon et al., 2009)	578	2.90	7.89
29	PTD04703.1	15	Superoxide dismutase [Cu-Zn]	With the cofactors destroys superoxid anion radicals (Jeong et al., 2001)	203	9.31	90.8
30	PTD06654.1	24	Superoxide dismutase [Mn], mitochondrial		475	0.73	15.32
31	PTD03720.1	58	Woronin body major protein	Required for survival and proper development (Soundararajan et al., 2004)	550	100.54	135.65
32	PTD11016.1	35	Malate dehydrogenase, mitochondrial	Responsible for pyruvate metabolism (M.A. Kwon et al., 2009; S.J. Kwon et al., 2009)	972	49.07	21.68
33	PTD07668.1	81	Peroxidase/catalase 2	Antioxidative role (Luhova et al., 2006)	151	0.81	10.76
34	PTD09346.1	64	Acid phosphatase		1090	1.33	8.29
35	PTD11648.1	14	Calmoduline	activate Ca ²⁺ signaling system	299	0.78	3.177
36	PTD11120.1	18	Translationally-controlled tumor protein	Binds Ca ²⁺ , lead to apoptosis (Berchtold and Villalobo, 2014)	124	1.69	5.58
37	PTD02437.1	24	Glutathione independent glyoxalase HSP31	Protect against ROS, catalyze the reaction of ethylglyoxal (MG) to D-lactate in a single GSH-independent step (https://www.uniprot.org/uniprot/Q04432 accession 27.01.2021)	682	3.25	14.67
38	PTD07292.1	24	Peroxioredoxin	Oxidative stress signaling	418	0.77	3.17
39	PTD13485.1	40	Putative oxidoreductase	Oxidoreductase activity	354	6.76	4.2

* ID – protein accession numbers.

** Mascot score – cumulative scores for individual peptides for all peptides matching a given protein.

*** Spot volume – the sum of the pixel intensities within the spot area.

cytoplasm, which enables microtubule stabilization, calcium-binding activities, and apoptosis (Kwon et al., 2019) and calmodulin, which is a calcium-binding messenger, participating in the signal transduction pathway (Masada et al., 2012). Hoshino et al. (1991) studied the Ca^{2+} signaling system in *F. oxysporum* and reported that when the concentrations of Ca^{2+} activated by calmodulin and dependent protein kinase increased, lipases were released externally.

Among the proteins with oxidation–reduction functions, an important role is played by GSH-independent glyoxalase HSP31, which is involved in protection against reactive oxygen species (ROS) and may negatively affect TORC1 in response to nutrient limitation (Hasim et al., 2014), and mitochondrial protein—peroxiredoxin, which transmits the signals as a response to oxidative stress (Cox et al., 2009), which were expressed in the extract treated sample. The study focused on the proteins which participate in the enzymatic ROS scavenging mechanism: peroxidase, CAT and SOD (Dąbrowska et al., 2012), higher amounts of which were detected in the extract treated culture.

The stress protein was expressed in a high volume in both systems - control (spot volume - 422) and in the extract-treated culture (505). The present study showed that the Woronin body major protein was expressed in the extract-treated culture. It is considered that these organelles are required for the survival and proper development of filamentous fungi. In addition, the Woronin body protects against nutrient restriction (Soundararajan et al., 2004).

3.2.5. PC/PE ratio and GSH concentration

In our previous papers (Bernat et al., 2018; Szewczyk et al., 2020), the effect of compounds inducing oxidative stress on the lipid profile and the functioning of microbial membranes was observed. Therefore, a decision was made to measure the changes in the cell membrane composition. Phospholipids are essential for the formation and stability of lipoproteins. PC and PE are the main building blocks of biological membranes and aid in the folding of the membrane proteins (Patel and Witt, 2017). After 24 h, an about 25 % decrease in the PC/PE ratio was noticed in the *T. harzianum* extract-treated sample compared to the control, which was maintained until the end of the culture, possibly indicating an increase in the stiffness of the fungal membrane (Bernat et al., 2018).

The total percentage of phospholipids was measured, and changes in the phosphatidylcholine (PC)/phosphatidylethanolamine (PE) ratio were revealed (Fig. 5); the results showed the lower ratio of these two groups of phospholipids during the entire breeding period in the extract-treated culture. Both classes accounted for approximately 90 percent of the phospholipids determined. The remaining phospholipids species were of the following classes: phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylserine (PS) (Supplementary Fig. 3).

The changes observed in the amount of phospholipids might have been related to the oxidative stress and membrane modifications (Singh

et al., 2010). In addition, the total concentration of GSH was measured, and the results indicated a lower content of these molecules in the extract-treated sample.

The important role of GSH in detoxifying cells has been widely described in previous studies (Staba et al., 2015). Moreover, it is also known that the deficit of GSH increases the oxidative stress in fungi (Oliveira-Silva et al., 2019).

Considering the obtained results, in the next part of the study, the level of oxidative stress was investigated by measuring the level of the antioxidant enzymes and identifying ROS.

3.2.6. CAT and SOD activity

During the previous analysis, four main periods were identified when changes of growth were taking place, during which CAT and SOD activity was measured. The results thus obtained (Fig. 6) show that the culture treated with *Trichoderma* extract exhibited a significant change in the production of the enzymes, during all 10 days of cultivation.

The first wave of enzymes activity was noticed beginning from 24 h—when a higher SOD activity was observed, compared to the control culture, and the other time points. According to Yao et al. (2016), metalloenzymes such as SOD become active as a first line of defense against ROS, which dismutates the superoxide anion. Then other enzymes, such as CAT detoxify hydrogen peroxide. During the second wave of enzymes activity, observed at 120 h, the highest CAT activity was found in the extract treated culture. From 168 h to the end of the cultivation, the decrease in the activity of these enzymes was noticed.

ROS were measured at two time points, with the highest activity of CAT and SOD enzymes. First, a higher level of O_2^- was noted at 120 h, and the level of H_2O_2 was slightly higher compared to the control. At 168 h, a significant increase in O_2^- was observed in the extract-treated sample, compared to the control. The H_2O_2 level did not differ from the previous measurement. The ROS scavenging mechanism is divided into an enzymatic and nonenzymatic system (Yao et al., 2016). The second one comprises small molecules like GSH that remove oxidants from solution. According to Fig. 5, GSH amounts were lower in the extract treated sample at 120 and 168 h, when the ROS activity was higher. These may have been correlated to the oxidative stress in *F. culmorum* culture (Phaniendra et al., 2015) caused by the presence of *Trichoderma* metabolites.

4. Conclusion

Summing up, the metabolites contained in the *T. harzianum* extract were found to inhibit the growth of *F. culmorum* on PDA plates, negatively affecting sporulation as well as inhibiting the production of dyes and main mycotoxin – zearalenone. In flask cultures, the metabolites caused severe oxidative stress and affected the ZEN production. The use of metabolomic and proteomic studies confirmed that the presence of metabolites produced by *T. harzianum* triggered significant changes in the physiology of *F. culmorum* at the molecular level. In addition, the obtained results showed the potential of compounds produced by *Trichoderma* fungi to act against plant pathogens. However, it is worth noting that the use of ZEN gene cluster analysis in future research could help explain some of the phenomena observed during the research, such as the increased production of mycotoxins in the presence of ethanol in the control systems.

Subsequent works can analyze whether *T. harzianum* extract can be used as a protective agent against *Fusarium* in a three-factor system involving the plant.

Authors contributions

JM Conceptualization, Investigation, Methodology, Writing – review & editing. SR Investigation, Methodology. AS – Methodology. PB – Investigation, Methodology, Funding acquisition, Writing – review & editing.

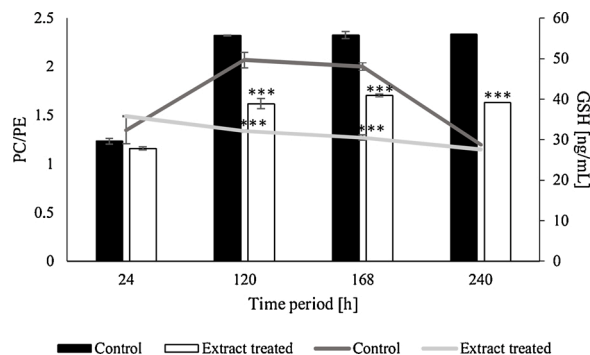


Fig. 5. PC/PE (bars) ratio and total GSH concentration (ng/mL) (curve) in the liquid culture of *F. culmorum* treated with the *T. harzianum* extract. Data represent mean \pm SE. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

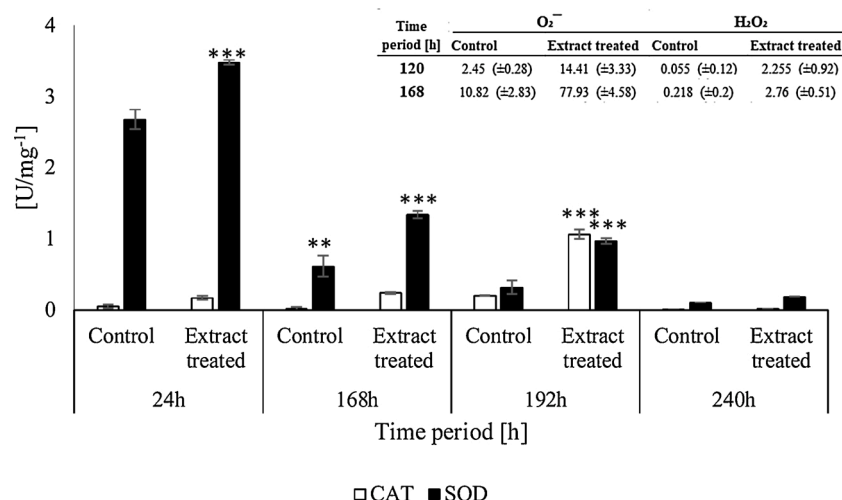


Fig. 6. Comparison of CAT and SOD activity in the liquid culture of *F. culmorum* treated with *T. harzianum* extracts at four time points. Data represent mean \pm SE. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Declaration of Competing Interest

All authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.micres.2021.126770>.

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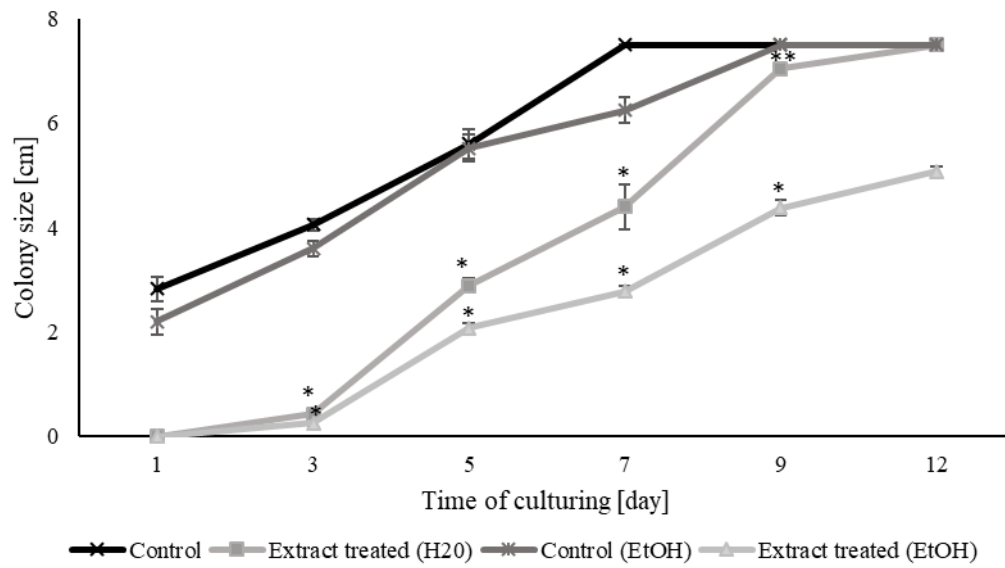
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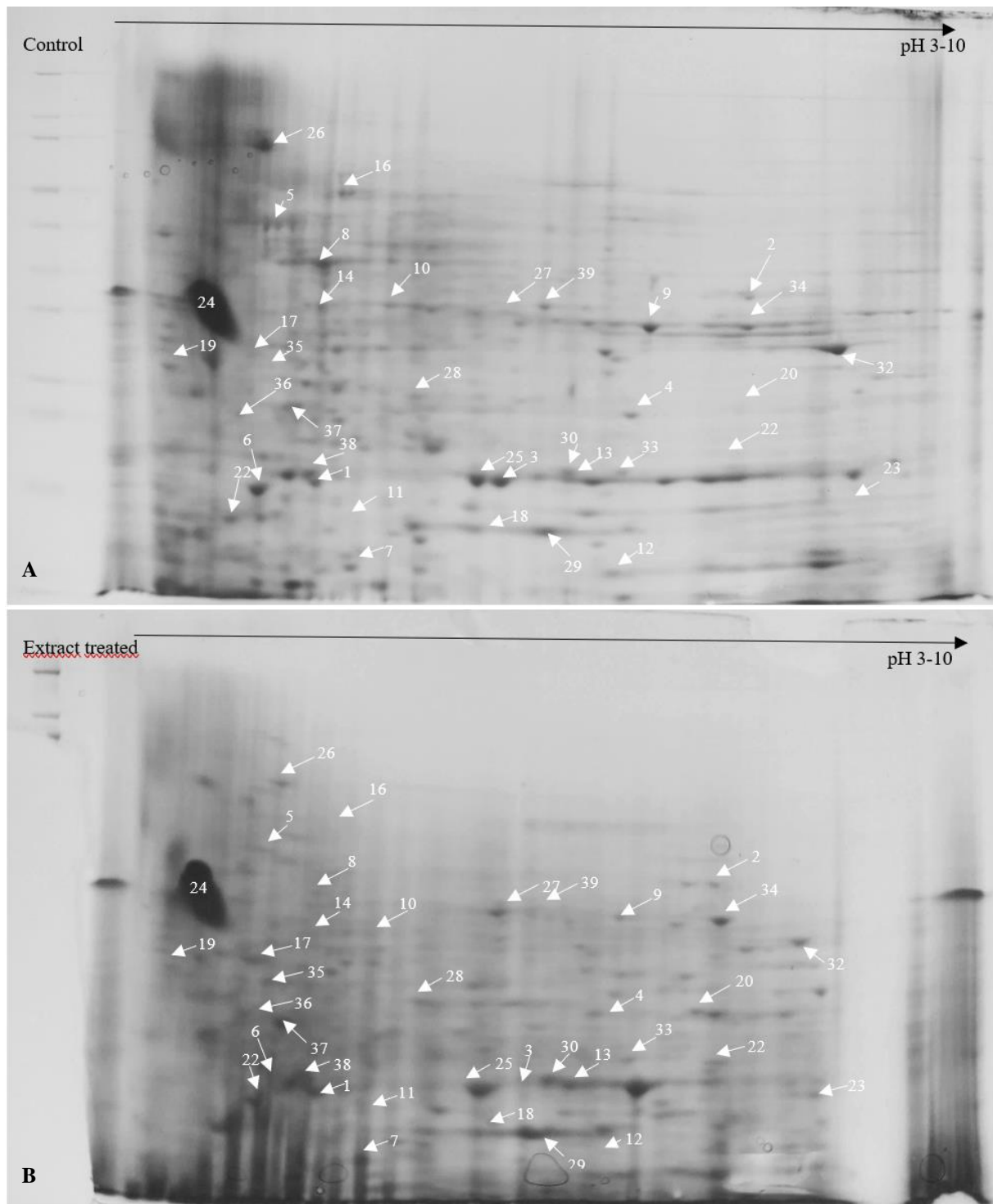
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P-2
Supplementary material

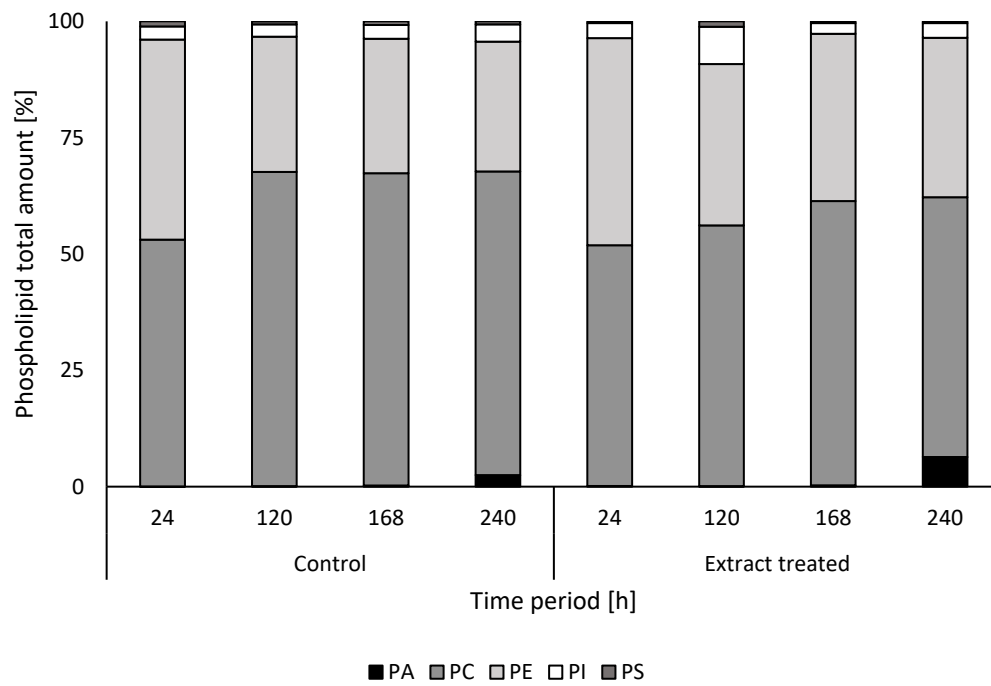
Supplementary material:



Supplementary Fig. 1. Comparison of colony growth on PDA plates, control culture (with H₂O or EtOH), and culture treated with *T. harzianum* extracts (dissolved in H₂O or EtOH). Data represent mean ± SE. * $p \leq 0.0001$, ** $p \leq 0.05$



Supplementary Fig. 2. 2-D SDS PAGE protein profile for control *F. culmorum* culture (A) and *T. harzianum* extract-treated *F. culmorum* culture (B).



Supplementary Fig. 3. Total phospholipids amount of *F. culmorum* grown in liquid culture treated with *T. harzianum* extract, compared to control.

S1 Table. Multiple reaction monitoring (MRM) transitions for phospholipids identified in *T. harzianum*.

Lipids	MRM transitions	
PA 16:0/18:2	671.5	255
PA 16:0/18:1	673.5	281
PE 16:0/18:3	712.5	277
PE 16:0/18:2	714.5	255
PE 16:0/18:1	716.5	281
PE 18:2/18:3	736.5	277
PE 18:2/18:2	738.5	279
PE 18:1/18:2	740.5	281
PE 18:1/18:1	742.5	281
PC 16:0/16:0	778.5	255
PC 16:0/18:2	802.5	279
PC 16:0/18:1	804.5	281
PC 18:1/18:3	826.5	277
PC 18:2/18:2	826.5	279
PC 18:1/18:2	828.5	279
PC 18:1/18:1	830.5	281
PI 16:0/18:2	833.5	279
PI 16:0/18:1	835.5	281
PI 18:2/18:2	857.5	279
PI 18:1/18:2	859.5	281
PS 18:1/18:2	784.5	279
PS 18:1/18:1	786.5	281
PS 18:2/18:2	782.5	279

Kolejny etap pracy doktorskiej obejmował dwa cele 1) na podstawie badań z zastosowaniem technik omicznych ocena zmian we wzroście i rozwoju pszenicy w obecności stresu abiotycznego (2,4-D) i biotycznego (*F. culmorum*) oraz *T. harzianum*; 2) porównanie skuteczność *T. harzianum* do wyekstrahowanych z podłoża płynnego zewnątrzkomórkowych metabolitów tego grzyba, dodanych do układu z rośliną.

Powyższy etap rozpoczęto od badań wzrostu i kiełkowania pszenicy, w tym celu pszenice badano w 12 różnych układach.

Układy obejmowały:

1. kontrolę – pszenicę z wodą,
2. pszenicę z 2,4D,
3. pszenicę z zarodnikami *T. harzianum*,
4. pszenicę z *T. harzianum* i 2,4-D,
5. pszenicę z zarodnikami *F. culmorum*,
6. pszenicę z *F. culmorum* i 2,4-D,
7. pszenicę z zarodnikami *T. harzianum* i *F. culmorum*,
8. pszenicę z *T. harzianum*, *F. culmorum* i 2,4-D
9. pszenicę z ekstraktem z płynu pohodowlanego *T. harzianum*
10. pszenicę z ekstraktem z płynu pohodowlanego *T. harzianum* z 2,4-D
11. pszenicę z ekstraktem z płynu pohodowlanego *T. harzianum* z zarodnikami *F. culmorum*
12. pszenicę z ekstraktem z płynu pohodowlanego *T. harzianum* z zarodnikami *F. culmorum* i 2,4-D.

Czas hodowli pszenicy dobrano eksperymentalnie – na podstawie szybkości wzrostu próby kontrolnej i ograniczone możliwości wzrostowe na szalkach - hodowlę prowadzono przez 7 dni. Kontrolowane warunki zapewniono przy użyciu fitotronu (IL/750/FIT P Pol-Eko, Polska) – 14h naświetlenia/ 10h w ciemności (intensywność światła $200 \mu\text{mol m}^{-2} \text{s}^{-1}$), wilgotność 40%. Przy optymalizacji parametrów hodowli, wyznaczono podlewanie pszenicy, co 24h z użyciem 1 ml destylowanej wody.

Po 7 dniach wykonano pomiary długości pędów i korzeni oraz oceniono kiełkowanie pszenicy. Najdłuższe pędy i korzenie odnotowano w próbie

z dodatkiem zarodników *T. harzianum*, kontroli i próbie z dodanym ekstraktem z płynu pohodowlanego *T. harzianum*. Wyniki te wskazują, że aktywność metabolitów *T. harzianum* jak i dodane zarodniki wytwarzające owe związki przyczyniają się do poprawy wzrostu pszenicy. Próby, do których dodano herbicyd, miały mniejszy rozwój systemu korzeniowego. Wśród pszenicy rosnącej w obecności dodanego patogenu odnotowano zahamowanie wzrostu zarówno pędów jak i korzeni, a także zauważono mniejszą zdolność do kiełkowania pszenicy (10-15%). Parametr ten został uległ poprawie po dodaniu *T. harzianum*, jak i ekstraktu z płynu pohodowlanego tego grzyba. Obserwując wzrost w dwóch najbardziej złożonych układach – nr.8 i nr.12 zauważono poprawę w kiełkowaniu w obecności ekstraktu z płynu pohodowlanego *T. harzianum*, natomiast długość pędów i korzeni była wyższa w układzie z dodanymi zarodnikami grzyba strzępkowego.

O stanie energetycznym rośliny decyduje jej potencjał wodny, który umożliwia transport wody w układzie rośliny z glebą i atmosferą [25]. Postanowiono, więc sprawdzić jak dodane do pszenicy czynniki stresowe wpływają na względną zawartość wody (RWC – ang. *Relative water content*). Dodanie do układów *F. culmorum* wpłynęło na obniżenie o ponad 30% RWC w porównaniu do układów kontrolnych. Dodanie zarodników *T. harzianum* do układu z zarodnikami *Fusarium*, nie wpłynęło na poprawę danego parametru, natomiast dodanie ekstraktu z płynu pohodowlanego *Trichoderma* powodowało, że spadek RWC nie był tak duży. RWC w całkowicie nabrzmiałych, zdrowych liściach wynosił powyżej 98% i poniżej 40% w więdnących, zamierających liściach [26].

Kolejnym badanym parametrem, była obecność mykotoksyny produkowanej przez wybrany szczep fitopatogenu. Opracowano metodę ekstrakcji metabolitów z sączków, umieszczonych w szalkach Petriego, na których kiełkowała pszenica oraz zastosowano celowaną metodę LC-MS/MS. Najwyższe stężenie oznaczono w układzie pszenicy z zarodnikami *F. culmorum*. Dodanie do układu zarodników *T. harzianum* wpłynęło na niższą zawartość badanego związku, co mogło być wynikiem konkurencji o składniki, które są niezbędne *Fusarium* do produkcji mykotoksyn [27]. Najmniejsze stężenie zearalenonu odnotowano w próbach zawierających ekstrakty z płynu pohodowlanego *T. harzianum*, wynik

ten nawiązuje do badań wykonanych w drugim etapie pracy doktorskiej (w bardziej złożonym układzie).

Jako wskaźnik stresu, na który narażana była pszenica w układach użyto do oznaczeń kwas jasmonowy syntetyzowany z cyklicznych kwasów tłuszczowych w reakcji obronnej rośliny na czynniki stresowe [28]. Dodanie herbicydu do układów znacząco podnosiło zawartość kwasu jasmonowego w pędach pszenicy, dodane zarodniki *Fusarium* również przyczyniły się do wyższego stężenia badanego hormonu. Pszenica pochodząca z układów z dodanym ekstraktem z płynu pohodowlanego *T. harzianum* miała niższy poziom stresu, nawet w układach z fitopatogenem.

Kolejnym ważnym badaniem, przeprowadzonym w trzecim etapie pracy doktorskiej, było określenie różnic w profilu białkowym pędów i korzeni każdego z układów. Etap rozpoczęto od optymalizacji metody ekstrakcji. Problem stanowiła niewielka ilość tkanki roślinnej otrzymanej po 7 dniach prowadzonej hodowli. Podjęto próby dostosowania objętości rozpuszczalników dla wydajnego procesu ekstrakcji. Po otrzymaniu zadawalającego stężenia białka, przeprowadzono analizę porównawczą 2D. Różnice w intensywności spotów identyfikowano za pomocą programu do analizy żeli SameSpot (Producent). W wyniku przeprowadzonej analizy odnotowano, że większość oznaczonych białek pochodząca z układów z czynnikami stresowymi była zaangażowana w procesy antyoksydacyjne i odpowiedzi na stres, a pochodząca z układów kontrolnych, z zarodnikami *T. harzianum* oraz ekstraktem z płynu pohodowlanego tego grzyba - w metabolizm węglowodanów czy procesy glikolityczne.

W korzeniach pszenicy zaszczerpionych zarodnikami *Fusarium*, z dodaniem 2,4-D stwierdzono większe ilości białek i enzymów pełniących funkcje oksydoredukcyjne, takich jak katalaza, peroksydaza askorbinianowa, peroksydaza cytochromu C i dysmutaza ponadtlenkowa Cu/Zn. *Trichoderma* i jej metabolity obecne w układach z zarodnikami *Fusarium* powodowały obniżenie intensywności spotów białek do wartości porównywalnych do kontroli. Przeprowadzono uzupełniające badanie z pomiarem SOD i CAT w pędach i korzeniach. Jako że głównym miejscem interakcji rośliny z mikroorganizmami jest korzeń, w 7 dniu stwierdzono wyższą zawartość SOD w układach

z zarodnikami *F. culmorum* a także w układach z 2,4-D. Przechodząc do badań drugiej linii obrony enzymatycznej przeciwko RFT, zauważono tylko niewielki wzrost stężenia katalazy w próbach z zarodnikami *Fusarium*.

W pędach oznaczono białka zaangażowane w fotosyntezę i reakcję na stres oksydacyjny. W układach z metabolitami i zarodnikami *T. harzianum* dominowały: aktywaza beta RUBISCO, białko wzmacniające wydzielanie tlenu (OEE) oraz karboksylaza 1,5-bisfosforanu rybulozy. Najmniejsze intensywności tych spotów stwierdzono w obecności *Fusarium* i 2,4-D. Białka OOE są niezbędne do fotosyntezy i biorą udział w fotooksydacji wody podczas reakcji świetlnych [29]. Otrzymany wynik ściśle korelował ze zbadanym stężeniem chlorofilu, oznaczonym za pomocą urządzenia Chlorophyll Content Meter CCM-300 (Opti-Sciences (Hudson, NY, USA)) w pędach pszenicy. Najwyższe stężenie chlorofilu stwierdzono w układach z zarodnikami *T. harzianum*, trzykrotnie niższe w układach z zarodnikami *F. culmorum* i 2,4-D. Dodanie ekstraktów z płynu pochodzącego z *T. harzianum* nie wpłynęło na zwiększenie zawartości chlorofilu, a nawet przyczyniło się do lekkiego obniżenia w układach bez czynników stresowych w porównaniu do kontroli.

Ostatnim etapem pracy doktorskiej było porównanie możliwości wykorzystania ekstraktów pochodzących z płynu pochodzącego z *T. harzianum*, zawierających m.in. kwas harzianowy w stosunku do użycia zarodników *T. harzianum*.

Podsumowując wyniki badań ujętych w trzecim etapie pracy stwierdzono, że dodanie ekstraktów z płynu pochodzącego z *T. harzianum* pozytywnie oddziałuje na kiełkowanie i rozwój pszenicy w obecności czynników stresowych w początkowych fazach rozwoju, natomiast dodanie zarodników tego grzyba okazuje się być efektywniejsze przez cały okres hodowli.

W ostatniej części pracy doktorskiej zastosowano dziesięć różnych metod badawczych, łącznie użyto 5 technik. Otrzymane wyniki opublikowano w czasopiśmie International Journal of Molecular Sciences (IF: 5,924; MNiSW: 140) pod tytułem “Potential of *Trichoderma harzianum* and Its Metabolites to Protect Wheat Seedlings against *Fusarium culmorum* and 2,4-D”.

P-3

“Potential of Trichoderma harzianum and Its Metabolites to Protect Wheat Seedlings against Fusarium culmorum and 2,4-D.”

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Article

Potential of *Trichoderma harzianum* and Its Metabolites to Protect Wheat Seedlings against *Fusarium culmorum* and 2,4-D

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Abstract: Wheat is a critically important crop. The application of fungi, such as *Trichoderma harzianum*, to protect and improve crop yields could become an alternative solution to synthetic chemicals. However, the interaction between the fungus and wheat in the presence of stress factors at the molecular level has not been fully elucidated. In the present work, we exposed germinating seeds of wheat (*Triticum aestivum*) to the plant pathogen *Fusarium culmorum* and the popular herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in the presence of *T. harzianum* or its extracellular metabolites. Then, the harvested roots and shoots were analyzed using spectrometry, 2D-PAGE, and MALDI-TOF/MS techniques. Although *F. culmorum* and 2,4-D were found to disturb seed germination and the chlorophyll content, *T. harzianum* partly alleviated these negative effects and reduced the synthesis of zearalenone by *F. culmorum*. Moreover, *T. harzianum* decreased the activity of oxidoreduction enzymes (CAT and SOD) and the contents of the oxylipins 9-Hode, 13-Hode, and 13-Hotre induced by stress factors. Under the influence of various growth conditions, changes were observed in over 40 proteins from the wheat roots. Higher volumes of proteins and enzymes performing oxidoreductive functions, such as catalase, ascorbate peroxidase, cytochrome C peroxidase, and Cu/Zn superoxide dismutase, were found in the *Fusarium*-inoculated and 2,4-D-treated wheat roots. Additionally, observation of the level of 12-oxo-phytodienoic acid reductase involved in the oxylipin signaling pathway in wheat showed an increase. *Trichoderma* and its metabolites present in the system leveled out the mentioned proteins to the control volumes. Among the 30 proteins examined in the shoots, the expression of the proteins involved in photosynthesis and oxidative stress response was found to be induced in the presence of the herbicide and the pathogen. In summary, these proteomic and metabolomic studies confirmed that the presence of *T. harzianum* results in the alleviation of oxidative stress in wheat induced by 2,4-D or *F. culmorum*.



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Keywords: plant elicitor; *Fusarium culmorum*; *Trichoderma harzianum*; proteomic study; antioxidative capacity

1. Introduction

Nowadays, crops are exposed to numerous stress factors—natural ones, caused by the presence of pests and synthetic ones, related to the use of protective agents such as pesticides. One of the important problems of agriculture worldwide are soil borne diseases. The presence of pathogens and chemical stressors may affect the quality of crops and cause economic losses. Recently, biological control agents (BCAs) with reduced environmental impact have become an alternative to synthetic pesticides [1]. The innovative method of using bacteria and fungi to induce plant resistance to abiotic stress has been actively studied in recent years [2].

Trichoderma spp. are soil-inhabiting filamentous fungi, including species with antagonistic activity against plant pathogens, such as *Pythium* spp., *Fusarium* spp., and others. The process of the pathogen growth inhibition depends on various mechanisms, such as

production of lytic enzymes and antibiotics and much faster space growth and nutrient consumption [3]. *Trichoderma* species are well known for their ability to produce various metabolites with antibiotic activity including peptaibols, polyketides, polypeptides, pyrones, and terpenes.

Fusarium species are a group of dangerous cereal pathogens (FHB—*Fusarium* head blight) that produce metabolites belonging to important mycotoxins. The most toxic metabolites produced by these fungi include nivalenol and zearalenone (ZEA), which are harmful to animals and people [4].

In our previous work, the impacts of 14-aminoacids peptaibols and the metabolites T22-azophilone and harzianic acid in *T. harzianum* extracellular extract on *Fusarium culmorum* growth and development together with zearalenone production were studied [3]. After their ability to reduce *F. culmorum* growth and ZEA production had been found, it was decided to continue research using both *T. harzianum* and its metabolites to prevent the development of the pathogen in the plant–fungal system with wheat.

According to our knowledge, *T. harzianum* can improve the germination of wheat kernels and reduce the toxic effects caused by the herbicide 2,4-D [5]. This strain is used as a commercial bioactive agent due to its ability to increase plant resistance against abiotic stresses and protect plants against pathogens [6] by competing with them for ingredients and even by parasitizing them [7].

Furthermore, 2,4D is a synthetic herbicide that mimics the natural hormone auxin, causing faster plant growth and leaf shedding. It was the first commercial herbicide to become popular because of its low cost, high efficacy, and selectivity. Crops such as wheat, rice, and barley are also susceptible to 2,4D's negative effects. The changes may include twisting of the stems and leaves, deformation of the head, deformation of the roots, and inhibition of growth [8]. Additionally, 2,4-D may cause overproduction of reactive oxygen species (ROS) and lipid peroxidation [9].

Based on our previous research, in the present study we hypothesized that *T. harzianum* could be an effective agent by reducing different stresses exerted on plant germination. *F. culmorum* was chosen as a biological stress factor and 2,4-D as an additional chemical stress factor. To verify this hypothesis, the experiment was conducted with wheat (*Triticum aestivum*) as a model plant, as it is sensitive to 2,4-D and *F. culmorum*. Germinated wheat was exposed to *T. harzianum* and its extracellular metabolites, *F. culmorum* and 2,4-D. Multiple molecular biology techniques were used to assess the impacts of *T. harzianum* on the development of plants in the early stages of germination in the presence of stress factors, involving a proteomic study of the shoots and roots and analysis of the oxidoreductive enzymes' activity and effects on jasmonic acid synthesis via ZEA determination.

2. Results and Discussion

2.1. Plant Germination and Growth Condition

In the present study, changes in the germination of wheat treated with different mixes of soil fungi with and without 2,4-D were investigated. To analyze how *Trichoderma* metabolites might act in a combined system of biological and chemical stress factors, *Trichoderma* metabolites extracts as well as *T. harzianum* fungus were used in the experiments.

To indicate changes taking place in the multivariate system, the first analysis was focused on plant germination and growth. The measurements were performed on the 7th day of cultivation (Figure 1).

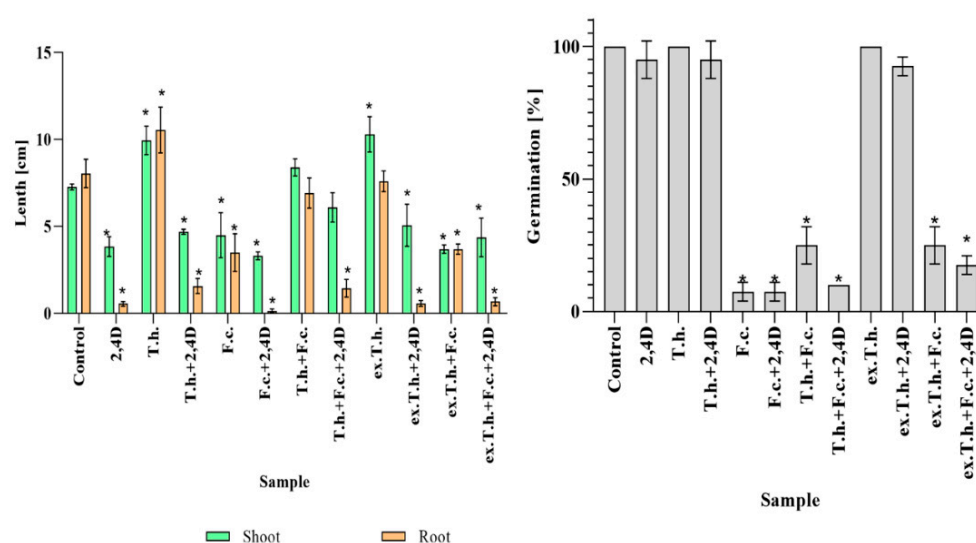


Figure 1. The total germination % values and the lengths of the seedlings measured after the 7th day of cultivation (T.h.—*T. harzianum*-inoculated wheat seeds; F.c.—*F. culmorum*-inoculated wheat seeds; ex.T.h.—*T. harzianum* extracellular metabolite-treated wheat seeds). Data represent means \pm SE; the significance of the variance study with Tukey's post hoc test = * $p \leq 0.001$. The subcolumn statistic comparison is attached in supplementary material as Table S1.

The 2,4-D added to each of the tested systems had negative impacts on root development and shoots. When *T. harzianum* fungus was added, the growth of the stressed wheat roots improved. Both *Trichoderma* fungus and its metabolites had positive effects on wheat shoot length. *F. culmorum* added to the examined cultures inhibited the seeds development, while the presence of *T. harzianum* mitigated the negative impacts of the pathogen. The behavior of the systems after the addition of the herbicides and *Fusarium*, in which *Trichoderma* extract was used, was comparable with the control system. This might have been due to a limitation in the development of *Fusarium*, as had been previously observed on PDA plates [3].

In the present study, it was revealed that *F. culmorum* added to the wheat seeds had a huge impact on germination, causing at least a 70% inhibition. Similarly, Kaur et al. described wheat susceptibility to *Fusarium* infection. A decrease in winter wheat yield was noted in South Dakota, where seed germination and yield loss reached 80% [10].

Trichoderma species are well known biocontrol agents that promote plant growth. In the study by Bernat et al., the *Trichoderma* strain was able to reduce the impacts of herbicides on shoots and roots [5]. Usage of its metabolites alone in the presence of the herbicide was not sufficient for root development to a degree comparable to that caused by the fungus.

2.2. Changes in Relative Water Content (RWC)

The energy status of a plant is determined by its water potential, which enables the transport of water in the soil–plant–atmosphere system [6]. In the present study, we decided to investigate how these stress factors would influence the basic parameters of wheat (Figure 2). In the absence of any wetting factors, the RWC in fully turgid transpiring leaves was found to reach above 98% and approx. 30–40% in wilting–dying or dried leaves [11].

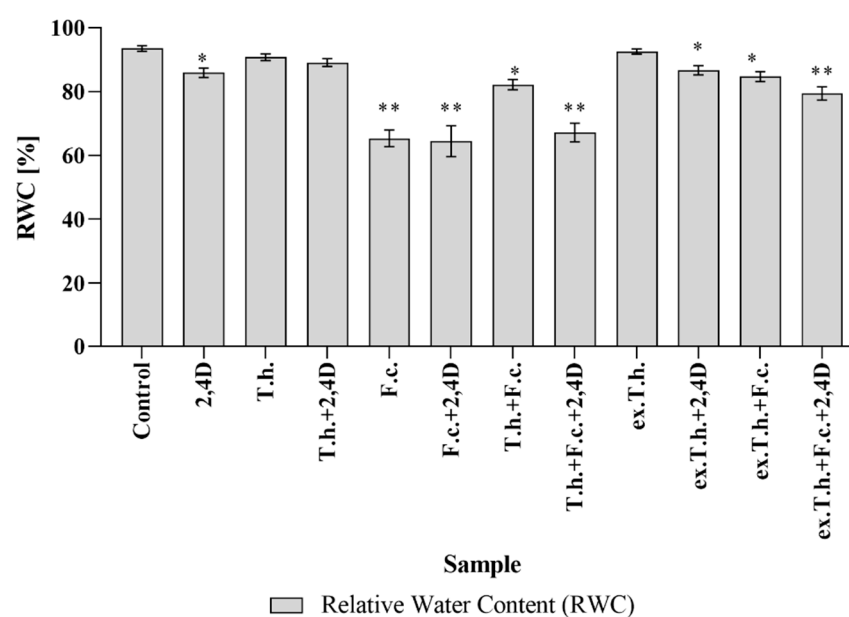


Figure 2. The RWC values of the shoot seedlings measured after 7 days of cultivation (T.h.—*T. harzianum*-inoculated wheat seeds; F.c.—*F. culmorum*-inoculated wheat seeds; ex.T.h.—*T. harzianum* extracellular metabolite-treated wheat seeds). Data represent means \pm SE; the significance of the variance study with Tukey's post hoc test = * $p \leq 0.001$, ** $p \leq 0.0001$. The subcolumn statistic comparison is attached in supplementary material as Table S2.

F. culmorum added to the wheat culture significantly reduced the water content in shoots by up to 65% compared to uninfected control (~93%). Pshibytko et al. (2006), who studied tomato wilt caused by *Fusarium* species, revealed that the RWC decreased during *Fusarium* wilt development [12].

A lower value of RWC in the presence of the pathogen was noted, whereas *T. harzianum* and its metabolites contributed to an increase in the RWC value. The usage of *Trichoderma* extracts gave the best result in a system with chemical and biological stress factors. According to Mohapatra and Mitra, who studied the potential of *Trichoderma viride* to prevent the oxidative stress caused by *Fusarium oxysporum* in wheat, co-infected seedlings showed significant reinforcement of RWC values in comparison with *Fusarium*-infected seedlings [13].

2.3. Changes in Chlorophyll Content

The amount of chlorophyll present in the leaves depends on the nutritional status of the plants. It is known that a deficiency of some minerals disrupts the development of chloroplast pigments in general and chlorophyll in particular [14].

Martinez et al. [15], who studied the dependency between the concentration of chlorophyll and the leaf turgor in wheat, revealed that RWC did not cause differences in the chlorophyll content, which was also confirmed by the results obtained in the present study (Figure 3).

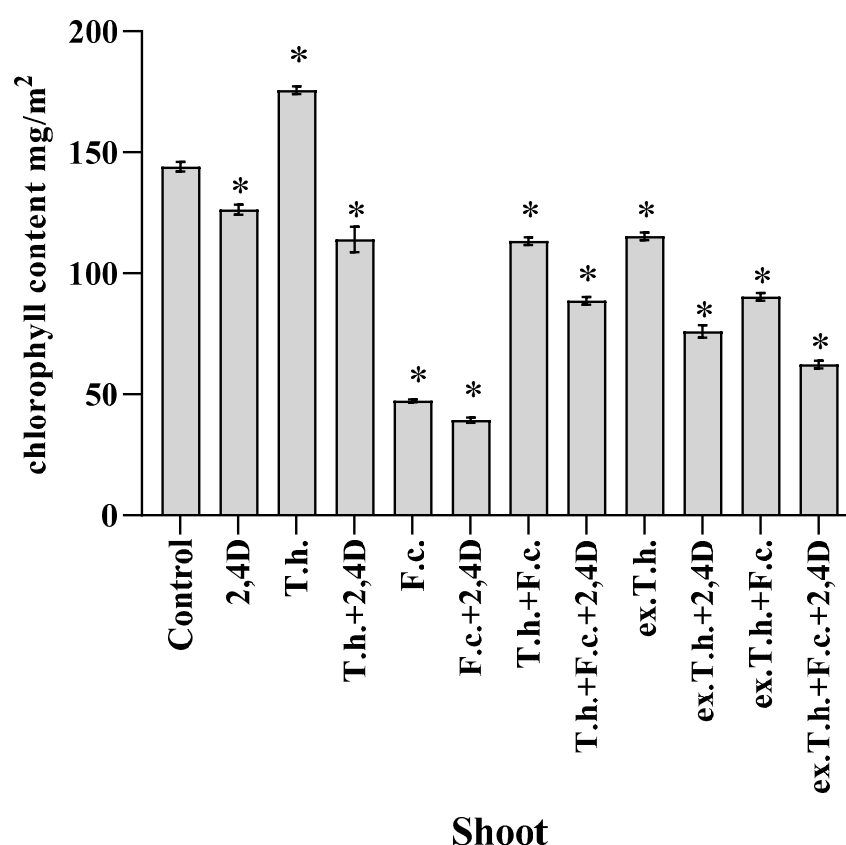


Figure 3. Chlorophyll contents in shoots measured after 7 days of cultivation (T.h.—*T. harzianum*-inoculated wheat seeds; F.c.—*F. culmorum*-inoculated wheat seeds; ex.T.h.—*T. harzianum* extracellular metabolite-treated wheat seeds). Data represent means \pm SE; the significance of the variance study with Tukey's post hoc test = * $p \leq 0.01$. The subcolumn statistic comparison is attached in supplementary material as Table S3.

Both the seedlings infected with *F. culmorum* and those treated with 2,4-D and the pathogen had the lowest concentrations (up to 50 mg/m²) of chlorophyll compared to the other wheat systems. Such reductions in chlorophyll content, which depend on the membrane stability, can be expected under stress conditions [16].

The seedlings infected by *Trichoderma* contained more chlorophyll. Even in contact with the studied stress factors, usage of the extract was not as effective. These results are in agreement with the findings of Zhang et al., who studied wheat grains infected with *T. longibrachiatum*, which contributed to the increased content of chlorophyll compared to the control system [17].

2.4. Changes in Jasmonic Acid

Jasmonic acid is responsible for the development and regulation of diverse plant defense responses. The molecules are derived from cyclic fatty acids and synthesized in response to various stressors [18].

During the analysis of early germinated shoots of wheat, different amounts of JA were noted (Figure 4). The pathogen inoculated on the seeds raised the content of the studied hormone compared to the control system. It is known that fungal infection is accompanied by changes in the amounts of two main hormones, salicylic acid (SA) and jasmonic acid, as well as the activation of SA and JA signaling [19].

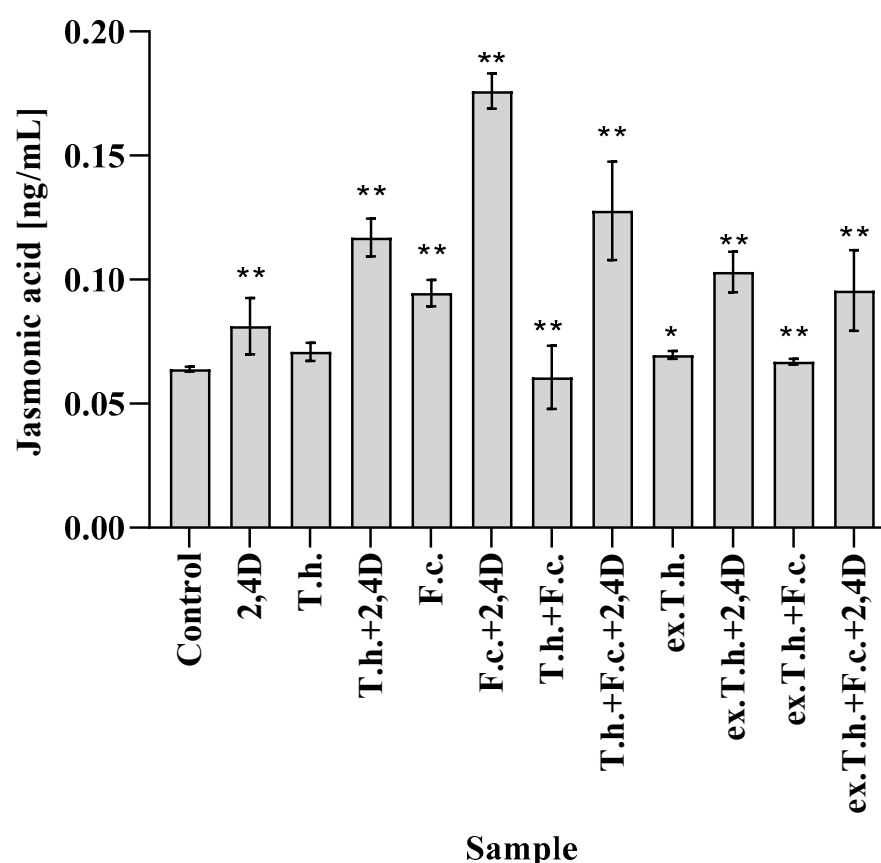


Figure 4. The contents of jasmonic acid in shoots measured after 7 days of cultivation (T.h.—*T. harzianum*-inoculated wheat seeds; F.c.—*F. culmorum*-inoculated wheat seeds; ex.T.h.—*T. harzianum* extracellular metabolite-treated wheat seeds). Data represent means \pm SE; the significance of the variance study with Tukey's post hoc test = * $p \leq 0.01$, ** $p \leq 0.001$. The subcolumn comparison is attached in supplementary material as Table S4.

Herbicide treatment of wheat resulted in only a slight lift in the level of the studied hormone. An approximately three-fold higher JA content (0.18 ng/mL) compared to the control system (0.06 ng/mL) was observed in the culture with the addition of both stress factors. The application of *Trichoderma* fungus reduced the hormone levels in the presence of *Fusarium*, although in the system with two stress factors the efficacy was very low. *T. harzianum* added to the wheat grains slightly increased the activity of JA, which is compatible with the findings of Moran-Diez et al., who studied the responses of tomato plants to the application of different *Trichoderma* species against *Pseudomonas syringae* [20].

It is known that increasing amounts of JA induce the expression of enzymes involved in the breakdown of chlorophyll [21]. There is a dependency between the amount of the studied hormone and the chlorophyll content (Figure 3)—the higher hormone concentration, the lower chlorophyll content. The exception was the system with *T. harzianum* and 2,4-D, in which a fairly high content of chlorophyll was noted.

2.5. Changes in Oxylipins

Jasmonic acid is an oxylipin hormone that is crucial for plants to regulate growth and stress response [21]. Oxylipins are formed in an enzymatic way but are also produced in response to singlet oxygen or reactive forms [22]. Taking into consideration these changes of the hormone content, the level of oxylipin in the shoots was measured. The testing was also conducted on the roots, as they are a major site of microorganism–plant interactions.

Trichoderma activity in the wheat system resulted in significant changes in the levels of the tested fatty acid derivatives in wheat shoots. In the presence of the herbicide, the levels

of 9-Hode and 13-Hode derived from linoleic fatty acids were reduced. The metabolites of *T. harzianum* added to the seeds controlled the 13-Hode level in the herbicide-treated system. A comparison of the cultures inoculated with *F. culmorum* showed significant growth of the oxylin levels in shoots, which was effectively reduced by the presence of *T. harzianum* or its extracts.

According to the obtained results (Figure 5), the presence of the herbicide induces the oxylin levels in the root systems. Differences in 9-Hode and 13-Hode of linoleic origin were observed, while 13-Hode from α -linolenic acid was also tracked. *F. culmorum* had the greatest impact on the derivatives of linoleic fatty acids. Seedlings exposed to *Trichoderma* or its metabolite activity demonstrated levels similar to the control system of the test oxylin. In the presence of 2,4-D or the pathogen, these levels were reduced. It had been previously revealed by our team that *T. harzianum* diminished oxidative stress caused by 2,4-D [5]. The effectiveness in reducing the stress caused by the pathogen action has not been studied yet.

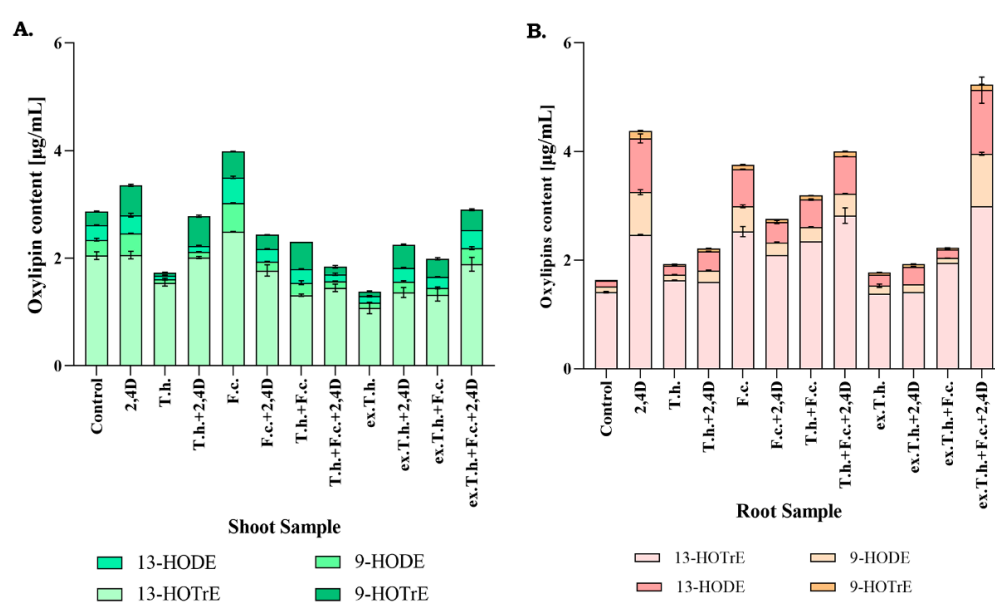


Figure 5. Relative oxylin amounts in the shoots (A) and roots (B) measured after 7 days of cultivation (T.h.—*T. harzianum*-inoculated wheat seeds; F.c.—*F. culmorum*-inoculated wheat seeds; ex.T.h.—*T. harzianum* extracellular metabolite-treated wheat seeds). Data represent means \pm SE; the significance of the variance study with Tukey's post hoc test. The subcolumn comparison is attached in supplementary material as Table S5.

2.6. Differences in Antioxidant Enzyme Activity

It is known that JA induces reactive oxygen species (ROS), which damage chloroplasts in the first target and induce senescence [21]. Additionally, due to the changes in the oxylin content, the activity levels of antioxidant enzymes in wheat shoots and roots were measured.

The mechanism of enzymatic decomposition of ROS proceeds in two steps—in the first step, superoxide dismutase is active, followed by the activation of catalase [22].

Taking into consideration the fact that 7 days wheat seedlings were used in the study, the main focus was on the first response step (Figure 6).

As the main interaction took place in the root system, the most pronounced changes were found in the enzyme activity in this part of the plant. The enzyme presence in the shoots might have been related to JA activity, causing the ROS induction.

The presence of the herbicide stimulated the activity of superoxide dismutase (SOD) in the roots of all studied samples. *T. harzianum* decreased the SOD activity in the systems, whether treated or untreated with 2,4-D. The addition of *F. culmorum* to wheat seedlings

resulted in extreme activation of the first-wave defense mechanisms against ROS. The highest activity (over 4 U/mg) was detected in the presence of chemical and biological stressors. In the presence of the studied biocontrol agent, the enzyme activity was lower, although it was not completely abolished.

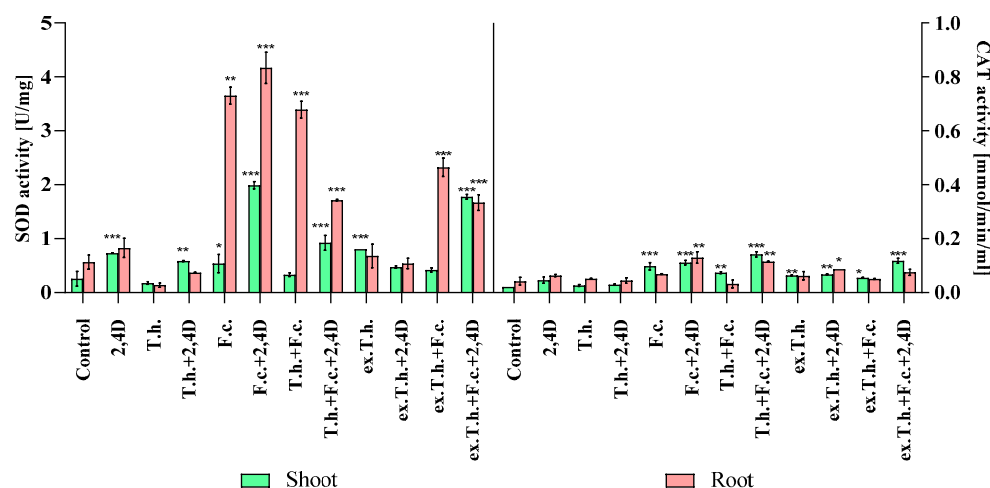


Figure 6. The oxidoreduction enzyme activity levels of the seedlings measured after 7 days of cultivation (T.h.—*T. harzianum*-inoculated wheat seeds; F.c.—*F. culmorum*-inoculated wheat seeds; ex.T.h.—*T. harzianum* extracellular metabolite-treated wheat seeds). Data represent means \pm SE; the significance of the variance study with Tukey's post hoc test = * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. The subcolumn statistic comparison is attached in supplementary material as Table S6.

The catalase (CAT) activity might not have revealed such significant differences because of the early stage of wheat development. Higher activity was noted in both plant organs in *Fusarium*-inoculated seeds in the presence of the herbicide.

Interesting findings were reported by Tančić-Živanovet al., who studied the effects of *Trichoderma* spp. on the antioxidant activity and growth promotion of pepper seedlings [23]. Positive correlations were found between activity levels of SOD, CAT, and other enzymes and germination in plants treated with the studied isolates. It could be possible that the high activity levels of these enzymes in stress-exposed and *Trichoderma*-inoculated systems had an effect on the amount of energy needed for growth. On the other hand, the activity was lower compared to the *Fusarium*- or 2,4-D-treated seeds. Perhaps, the spore concentration or time of growth should have been chosen differently to ensure better antioxidant effectiveness of *T. harzianum*.

2.7. Changes in Zearalenone Content

Zearalenone is one of the most toxic mycotoxins, which is produced by *Fusarium* species and exerts deleterious effects on the reproductive capacity of animals [24].

In order to eliminate the development and toxic effect of *F. culmorum*, *T. harzianum* and its metabolites were added to the wheat culture. The results presented above (Figure 7) show the changes in mycotoxin production by the pathogen inoculated on the grains. Under standard conditions, the production of ZEA was approximately 0.3 ng/mL, while the treatment with 2,4-D raised the production capacity to 0.5 ng/mL, which was the highest possible concentration. The herbicide might have supported the development of the pathogen by limiting the plant growth.

The presence of *T. harzianum* in the system with the pathogen resulted in weaker production of mycotoxin. It is known that for the effective production of mycotoxins, *Fusarium* species require nutrient availability [25]. The emergence of such a persistent competitor as *Trichoderma* limits the pathogen development and results in the activation of defense mechanisms. The most effective method turned out to be the addition of *T. harzianum* extracts, which were found to influence the development of the fungus [3].

This observation is consistent with the study by Gromadzka et al. [26], who showed that two various *Trichoderma* isolates reduced the amounts of ZEA produced by four different *Fusarium* strains in solid substrate bioassays.

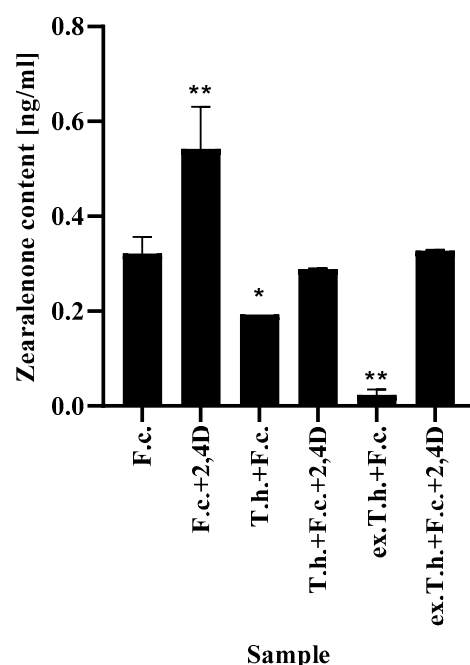


Figure 7. The ZEA levels in filter paper after 7 days of cultivation (T.h.—*T. harzianum*-inoculated wheat seeds; F.c.—*F. culmorum*-inoculated wheat seeds; ex.T.h.—*T. harzianum* extracellular metabolite-treated wheat seeds). Data represent means \pm SE; the significance of the variance study with Tukey's post hoc test = * $p \leq 0.01$, ** $p \leq 0.001$. The subcolumn statistic comparison is attached in supplementary material as Table S7.

Comparing the possible usage of *Trichoderma* or its metabolites, the second option is a much more effective method—from the very beginning the metabolites acted with *Fusarium*, interfering with the fungal development. The application of *T. harzianum* was less effective, because the fungi needed time to produce the metabolites externally, which gave a temporary opportunity for pathogen development.

2.8. Proteomic Study

There are two sensitive methods for protein analysis—MALDI TOF/TOF and LC-MS/MS. The first one with a very high processing capacity, the second, much more sensitive, capable of detecting several proteins in one spot [27].

The aim of proteomic study, included in present work, was not to interpret the entire protein map, but to look for the most pronounced changes that correlated with the previously obtained results.

Proteomic analysis based on estimation of the changes of proteins produced by the plant under different conditions was performed. The intensity values (spot volumes) were multiplied and spots with different volumes (the sum of the pixel intensities within the spot area) were excised out and digested for determination. A few spots were identified as being the same protein or representing isoforms. It is known that some spots contain several proteins, but analysis that was used in the present study have the lack in sensitivity compare to the LC-MS/MS [28], while the results given below show that the goal has been achieved, and our basic proteomics proved to be helpful in confirming the previously obtained results.

The results showed only the greatest changes detected in wheat shoots and roots (Table 1). SDS gels with marked spots are attached as Supplementary Figures S1–S4.

Table 1. Changes in average protein spot volumes, after SDS-Page electrophoresis and trypsin digestion analyzed by MALDI TOF/TOF identified by Mascot from root and shoot material of wheat, analyzed after 7 days of cultivation (T. h.—*T. harzianum* inoculated wheat seeds, F. c.—*F. culmorum* inoculated wheat seeds, ex. T. h.—*T. harzianum* extracellular metabolites treated wheat seeds).

Spot ID	Accession Number *	Theo-Retical MW [Da]	Score **	Protein	Function	Examined Avarege ROOT Sample Volumes ***												p Value ****
						Control	2,4D	T.h.	T.h.+ 2,4D	F.c.	F.c.+ 2,4D	T.h.+F.c.	T.h.+F.c.+ 2,4D	ex.T.h.	ex.T.h.+ 2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+ 2,4D	
1	AAB67990.1	20,310	106	Cu/Zn superoxide dismutase	Oxidoreductase/ response to oxidative stress	5.86 × 10 ⁴	2.53 × 10 ⁴	1.63 × 10 ⁴	7.07 × 10 ⁴	6.90 × 10 ⁵	1.56 × 10 ⁵	3.95 × 10 ⁵	4.88 × 10 ⁵	1.80 × 10 ⁴	1.88 × 10 ⁴	2.29 × 10 ⁵	2.83 × 10 ⁵	0.01
2	KAF7042201.1	26,606	493	Ascorbate peroxidases and cytochrome C peroxidases		1.14 × 10 ⁴	6.77 × 10 ³	9.58 × 10 ³	6.00 × 10 ⁴	6.31 × 10 ⁴	1.25 × 10 ⁵	1.09 × 10 ⁵	1.14 × 10 ⁵	1.83 × 10 ⁴	1.79 × 10 ⁵	3.87 × 10 ⁴	3.31 × 10 ⁴	0.03
3	KAF7054315.1	27,470	505	catalase/ peroksidase HPI		4.14 × 10 ⁴	9.32 × 10 ⁴	1.37 × 10 ⁴	6.32 × 10 ⁴	7.14 × 10 ⁴	1.72 × 10 ⁵	1.12 × 10 ⁵	1.35 × 10 ⁵	3.93 × 10 ⁴	8.35 × 10 ⁴	6.34 × 10 ⁴	5.24 × 10 ⁴	0.02
4	ACF70712.1	32,429	142	root peroxidase		1.73 × 10 ⁴	1.41 × 10 ⁴	5.06 × 10 ⁴	1.55 × 10 ⁴	8.83 × 10 ³	4.62 × 10 ³	6.93 × 10 ³	3.14 × 10 ³	6.54 × 10 ⁴	4.41 × 10 ⁴	5.13 × 10 ³	3.93 × 10 ³	0.007
5	KAF6989700.1	16,607	375	nucleoside diphosphate kinase		1.38 × 10 ⁵	4.98 × 10 ⁴	2.20 × 10 ⁵	2.08 × 10 ⁵	2.10 × 10 ⁴	4.14 × 10 ⁴	8.01 × 10 ⁴	7.09 × 10 ⁴	4.63 × 10 ⁴	3.51 × 10 ⁴	2.67 × 10 ⁴	2.06 × 10 ⁴	0.02
6	AAF64241.1	25,277	156	cytosolic glyceraldehyde-3-phosphate dehydrogenase GAPDH		8.58 × 10 ⁴	1.51 × 10 ⁵	7.68 × 10 ⁴	8.64 × 10 ⁴	9.05 × 10 ⁴	5.45 × 10 ⁴	8.91 × 10 ⁴	6.61 × 10 ⁴	6.03 × 10 ⁴	1.38 × 10 ⁵	1.17 × 10 ⁵	8.33 × 10 ⁴	0.01
7	CAI47635.1	36,482	107	peroxidase precursor		1.18 × 10 ⁴	1.24 × 10 ⁴	1.62 × 10 ⁴	3.65 × 10 ⁴	6.58 × 10 ⁴	4.20 × 10 ⁴	5.65 × 10 ⁴	9.05 × 10 ⁴	2.13 × 10 ⁴	1.63 × 10 ⁴	3.23 × 10 ⁴	3.96 × 10 ⁴	0.03
8	KAF6996030.1	33,096	114	NmrA-like family		5.02 × 10 ⁴	2.44 × 10 ⁴	6.37 × 10 ⁴	3.30 × 10 ⁴	9.64 × 10 ⁴	1.13 × 10 ⁵	1.04 × 10 ⁵	1.02 × 10 ⁵	1.62 × 10 ⁴	1.38 × 10 ⁴	9.83 × 10 ⁴	7.66 × 10 ⁴	0.03
9	AFC87832.1	40,578	80	12-oxo-phytodienoic acid reductase	Oxylipin biosynthesis	4.75 × 10 ⁴	1.86 × 10 ⁵	3.27 × 10 ⁴	1.89 × 10 ⁵	1.78 × 10 ⁵	1.15 × 10 ⁵	1.24 × 10 ⁵	1.42 × 10 ⁵	2.15 × 10 ⁵	1.79 × 10 ⁵	8.76 × 10 ⁴	1.36 × 10 ⁵	0.03
10	AAC23502.1	56,398	97	vacuolar invertase, partial	Carbohydrate metabolism	1.06 × 10 ⁴	7.49 × 10 ³	1.64 × 10 ⁴	1.25 × 10 ⁴	5.39 × 10 ³	8.02 × 10 ³	1.30 × 10 ⁴	8.08 × 10 ³	3.23 × 10 ³	4.99 × 10 ³	6.66 × 10 ³	5.64 × 10 ³	0.02
11	AGN71004.1	33,254	130	xylanase inhibitor protein precursor		1.18 × 10 ⁴	2.05 × 10 ⁴	7.92 × 10 ³	4.49 × 10 ³	1.15 × 10 ⁴	5.50 × 10 ³	5.18 × 10 ³	1.27 × 10 ⁴	6.75 × 10 ³	1.84 × 10 ³	1.63 × 10 ⁴	9.71 × 10 ³	0.03
12	ATY36097.1.	47,317	408	alpha-amylase		1.02 × 10 ⁵	1.34 × 10 ⁵	3.14 × 10 ⁵	2.23 × 10 ⁵	2.55 × 10 ⁵	1.16 × 10 ⁵	1.79 × 10 ⁵	1.49 × 10 ⁵	4.46 × 10 ⁵	2.70 × 10 ⁵	2.78 × 10 ⁵	1.88 × 10 ⁵	0.04
13	KAF6992161.1	24,332	525	malate dehydrogenase		8.16 × 10 ⁴	1.75 × 10 ⁵	1.87 × 10 ⁵	1.98 × 10 ⁵	1.63 × 10 ⁵	1.30 × 10 ⁵	1.46 × 10 ⁵	1.10 × 10 ⁵	9.40 × 10 ⁴	1.19 × 10 ⁵	2.36 × 10 ⁵	1.84 × 10 ⁵	0.03
14	AAX83262.1	28,242	83	class II chitinase		9.93 × 10 ⁴	4.25 × 10 ⁴	1.19 × 10 ⁵	4.78 × 10 ⁴	3.37 × 10 ⁵	2.89 × 10 ⁵	5.19 × 10 ⁵	2.40 × 10 ⁵	2.14 × 10 ⁵	1.20 × 10 ⁵	3.84 × 10 ⁴	4.33 × 10 ⁴	0.03
15	ABY85789.1	43,153	366	S-adenosylmethionine synthetase		3.03 × 10 ⁵	1.89 × 10 ⁵	1.94 × 10 ⁵	1.13 × 10 ⁵	1.62 × 10 ⁵	1.43 × 10 ⁵	2.00 × 10 ⁵	8.32 × 10 ⁴	1.48 × 10 ⁵	1.83 × 10 ⁵	1.96 × 10 ⁵	1.74 × 10 ⁵	0.02
16	CAC94001.1	24,984	324	glutathione transferase	Glutathione metabolic process	6.70 × 10 ³	3.71 × 10 ⁴	1.99 × 10 ⁴	1.74 × 10 ⁴	7.68 × 10 ³	2.32 × 10 ⁴	1.50 × 10 ⁴	2.67 × 10 ⁴	2.78 × 10 ⁴	1.35 × 10 ⁴	5.18 × 10 ³	7.73 × 10 ³	0.03
17	AAL71854.1	23,343	277	dehydroascorbate reductase		1.28 × 10 ⁴	1.45 × 10 ⁴	3.10 × 10 ⁴	1.16 × 10 ⁴	9.22 × 10 ³	8.29 × 10 ³	8.98 × 10 ³	1.50 × 10 ⁴	1.02 × 10 ⁴	7.24 × 10 ³	3.14 × 10 ³	4.60 × 10 ³	0.03
18	CAC14917.1	26,786	197	triosephosphat-isomerase		1.41 × 10 ⁵	1.02 × 10 ⁵	6.86 × 10 ⁴	1.31 × 10 ⁵	3.16 × 10 ⁵	3.49 × 10 ⁵	4.62 × 10 ⁵	3.44 × 10 ⁵	4.21 × 10 ⁴	1.13 × 10 ⁵	2.98 × 10 ⁴	2.31 × 10 ⁴	0.03
19	AVL25146.1	38,772	119	fructose-1,6-bisphosphate aldolase	Glycolytic process	1.37 × 10 ⁴	2.37 × 10 ⁴	5.92 × 10 ⁴	6.02 × 10 ⁴	1.23 × 10 ⁴	1.55 × 10 ⁴	2.65 × 10 ⁴	6.65 × 10 ⁴	1.64 × 10 ⁴	7946.144	4.29 × 10 ⁴	7.13 × 10 ⁴	0.03
20	AAP80633.1	29,558	265	phosphoglycerate mutase, partial		2.59 × 10 ⁴	3.16 × 10 ⁴	5.61 × 10 ⁴	2.06 × 10 ⁴	3.32 × 10 ⁴	4.40 × 10 ⁴	4.22 × 10 ⁴	3.22 × 10 ⁴	4.21 × 10 ⁴	4.81 × 10 ⁴	7.30 × 10 ⁴	6.48 × 10 ⁴	0.03
21	ALE18234.1	25,277	330	glyceraldehyde-3-phosphate dehydrogenase GAPDH		1.83 × 10 ⁶	1.33 × 10 ⁶	1.73 × 10 ⁶	1.66 × 10 ⁶	1.30 × 10 ⁶	1.10 × 10 ⁶	7.96 × 10 ⁵	1.23 × 10 ⁶	8.86 × 10 ⁵	1.07 × 10 ⁶	1.08 × 10 ⁶	9.68 × 10 ⁵	0.02
22	AWS00780.1	41,701	151	actin	ATP-binding	3.84 × 10 ⁴	3.14 × 10 ⁴	3.83 × 10 ⁴	2.00 × 10 ⁴	3.24 × 10 ⁴	3.52 × 10 ⁴	4.30 × 10 ⁴	3.14 × 10 ⁴	1.98 × 10 ⁴	2.52 × 10 ⁴	4.93 × 10 ⁴	4.41 × 10 ⁴	0.04
23	AGN94842.1	73,171	597	ER molecular chaperone	ATPase activity	3.72 × 10 ⁴	8.52 × 10 ⁴	4.34 × 10 ⁴	3.70 × 10 ⁴	7.83 × 10 ⁴	3.89 × 10 ⁴	2.42 × 10 ⁴	2.60 × 10 ⁴	2.58 × 10 ⁴	8.12 × 10 ⁴	2.88 × 10 ⁴	4.69 × 10 ⁴	0.02
24	CAA52636.1	59,212	1090	ATP synthase beta subunit		5.10 × 10 ⁵	6.77 × 10 ⁵	4.63 × 10 ⁵	1.12 × 10 ⁵	1.53 × 10 ⁵	1.62 × 10 ⁵	1.72 × 10 ⁵	1.56 × 10 ⁵	3.25 × 10 ⁵	2.05 × 10 ⁵	1.46 × 10 ⁵	1.12 × 10 ⁵	0.001

Table 1. Cont.

Spot ID	Accession Number*	Theo-Retical MW [Da]	Score**	Protein	Function	Examined Average ROOT Sample Volumes***												p Value****
						Control	2,4D	T.h.	T.h.+ 2,4D	F.c.	F.c.+ 2,4D	T.h.+F.c.	T.h.+F.c.+ 2,4D	ex.T.h.	ex.T.h.+ 2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+ 2,4D	
25	ADK35122.1	14,182	203	profilin	Actin binding	8.30×10^4	3.16×10^4	6.87×10^4	5.20×10^4	2.54×10^4	3.99×10^4	3.72×10^4	2.60×10^4	1.03×10^4	2.28×10^4	1.08×10^4	1.83×10^4	0.03
26	AIZ95472.1	16,047	141	actin-depolymerizing factor 3		3.63×10^3	2.12×10^3	1.97×10^3	1.40×10^3	2.96×10^3	5.60×10^3	2.37×10^3	1.56×10^3	1.43×10^3	1.45×10^3	1.94×10^3	1.60×10^3	0.03
27	AAA75104.1	16,245	71	single-strained nucleic acid binding protein	RNA-binding	3.93×10^4	2.99×10^4	2.85×10^4	9.71×10^3	2.54×10^4	3.99×10^4	3.73×10^4	2.60×10^4	1.07×10^4	1.91×10^4	1.08×10^4	1.83×10^4	0.03
28	AAA80609.1	19,651	158	adenine phosphoribosyltransferase form 1	Adenine salvage	6.02×10^3	2.05×10^3	9.80×10^3	1.57×10^3	8.17×10^3	6.19×10^3	8.77×10^3	2.48×10^3	8.92×10^3	1.49×10^3	1.17×10^4	5.37×10^3	0.01
29	CAA46811.1	38,331	123	cathepsin	Regulation of catalytic activity	3.25×10^4	3.10×10^4	6.11×10^4	2.68×10^4	4.28×10^4	2.70×10^4	5.19×10^4	2.10×10^4	2.72×10^4	5.22×10^4	3.04×10^4	3.35×10^4	0.03
30	ABB80135.1	26,160	179	vacuolar proton ATPase factor 3	Ion transport	4.46×10^3	3.20×10^3	3.24×10^3	7.57×10^3	1.73×10^4	1.15×10^4	2.49×10^4	2.19×10^4	1.06×10^4	3.36×10^3	1.33×10^4	6.70×10^3	0.02
31	KAF7056598.1	82,549	193	5-methyltetrahydropteroyl-triglutamate homocysteine methyltransferase	Amino-acid biosynthesis	1.43×10^3	1.88×10^4	4.71×10^3	3.30×10^3	2.17×10^3	3.16×10^3	4.54×10^3	7.28×10^3	4.91×10^3	4.97×10^3	2.34×10^3	4.45×10^3	0.01
32	AAZ95171.1	17,352	162	eukaryotic translation initiation factor	Protein biosynthesis	6.13×10^4	9.50×10^4	1.93×10^4	5.32×10^4	3.92×10^4	4.93×10^4	8.33×10^4	7.09×10^4	1.61×10^4	2.23×10^4	4.35×10^4	3.33×10^4	0.03
33	AAS17067.1	18,367	228	cyclophilin A	Protein folding	3.27×10^5	5.52×10^4	5.13×10^4	1.24×10^5	5.43×10^4	6.52×10^4	9.64×10^3	4.07×10^4	5.94×10^4	9.39×10^4	5.12×10^4	3.71×10^4	0.04
34	ABQ51156.1	24,404	184	Triticin, partial	Storage protein	1.65×10^3	8.33×10^2	1.64×10^3	2.07×10^3	1.51×10^4	1.33×10^3	2.94×10^3	2.55×10^3	1.27×10^3	7.88×10^2	3.18×10^3	4.60×10^3	0.03
35	ADQ85915.1	15,293	125	abscisic stress-ripening protein	Stress response	4.02×10^3	6.95×10^3	2.21×10^4	8.49×10^3	2.71×10^3	5.48×10^3	1.17×10^4	3.43×10^3	5.58×10^3	2.21×10^3	3.07×10^3	4.06×10^3	0.02
36	ADN05856.1	65,057	78	HOP		6.88×10^3	9.09×10^3	2.01×10^4	7.43×10^3	6.42×10^3	3.40×10^3	6.16×10^3	1.14×10^4	1.25×10^4	1.46×10^4	1.93×10^3	3.86×10^3	0.03
37	KAF7107406.1	25,857	161	co-chaperonin		1.01×10^4	6.21×10^3	1.81×10^3	5.95×10^3	1.71×10^3	2.50×10^3	2.14×10^3	2.79×10^3	2.53×10^3	4.21×10^3	2.08×10^3	1.86×10^3	0.04
38	ACQ41884.1	23,369	212	germin-like protein		1.68×10^5	2.38×10^5	5.22×10^5	1.71×10^3	6.47×10^5	6.34×10^5	2.70×10^5	3.87×10^5	1.43×10^5	3.70×10^5	4.99×10^5	3.51×10^5	0.01
39	KAF7063064	32,946	78	allergenic/antifungal thaumatin-like proteins	Defence response	1.07×10^5	1.51×10^5	6.64×10^4	3.74×10^4	1.14×10^5	1.55×10^5	1.30×10^5	1.25×10^5	2.03×10^5	1.21×10^5	5.46×10^4	6.45×10^4	0.03
40	ABX89061.1	17,023	147	pathogenesis-related protein		1.65×10^3	9.42×10^2	2.18×10^3	1.07×10^3	3.66×10^3	2.86×10^3	1.43×10^3	9.59×10^2	6.73×10^2	2.95×10^3	7.48×10^2	7.57×10^2	0.02
41	AFC89429.1	42,969	77	serpin-N3.2	Endopeptidase inhibitor activity	5.11×10^3	5.73×10^3	1.98×10^4	4.96×10^3	1.24×10^4	2.89×10^4	1.15×10^4	4.72×10^3	2.99×10^3	5.77×10^3	7.34×10^3	3.79×10^3	0.03
101	AFF19563.1	20,310	138	superoxide dismutase	Oxidoreductase	2.55×10^4	6.75×10^3	7.35×10^3	7.31×10^3	3.11×10^4	4.78×10^4	2.18×10^4	2.68×10^4	4.71×10^3	8.16×10^3	4.42×10^4	4.06×10^4	0.01
102	CAI47635.1	36,482	146	peroxidase precursor		6.18×10^4	9.25×10^4	7.11×10^4	6.63×10^4	4.40×10^4	2.24×10^4	4.25×10^4	4.06×10^4	1.55×10^5	9.70×10^4	3.76×10^4	6.98×10^4	0.04
103	ACO90196.1	26,606	151	ascorbate peroxidase		2.60×10^4	1.29×10^4	5.55×10^3	5.47×10^4	3.09×10^4	5.13×10^4	4.23×10^4	1.35×10^4	1.32×10^4	4.29×10^4	1.13×10^4	4.66×10^4	0.001
104	CDX58685.1	41,427	155	RUBISCO activase beta		4.04×10^4	1.03×10^5	1.61×10^5	7.41×10^4	7.64×10^4	4.87×10^4	6.39×10^4	5.47×10^4	1.13×10^5	1.34×10^5	9.84×10^4	6.55×10^4	0.03
105	AAM88439.1	23,711	76	putative Rieske Fe-S precursor protein	Photosynthetic electron transport chain	2.41×10^4	8.57×10^3	2.52×10^4	2.84×10^4	2.46×10^4	6.06×10^4	5.65×10^4	5.00×10^4	1.18×10^4	1.29×10^4	4.71×10^4	3.58×10^4	0.01
106	ARQ82872.1	27,184	483	oxygen evolving enhancer protein		1.21×10^6	6.33×10^5	1.28×10^6	7.78×10^5	1.42×10^5	1.65×10^5	1.25×10^5	1.15×10^5	1.25×10^6	1.16×10^6	6.60×10^4	1.05×10^5	0.01
107	BAA35176.1	52,817	407	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit		3.57×10^5	3.24×10^5	5.41×10^5	5.32×10^5	4.02×10^4	4.80×10^4	4.22×10^4	6.83×10^4	2.28×10^5	1.82×10^5	9.59×10^4	3.96×10^4	0.04

Table 1. Cont.

Spot ID	Accession Number*	Theo-Retical MW [Da]	Score**	Protein	Function	Examined Avarege ROOT Sample Volumes***												p Value****
						Control	2,4D	T.h.	T.h.+ 2,4D	F.c.	F.c.+ 2,4D	T.h.+F.c.	T.h.+F.c.+ 2,4D	ex.T.h.	ex.T.h.+ 2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+ 2,4D	
108	CDX48684.1	44,554	312	RUBISCO activase alpha	Photosynthetic electron transport chain	3.12×10^4	6.98×10^4	4.68×10^4	2.32×10^4	4.20×10^4	5.16×10^4	4.80×10^4	3.97×10^4	4.88×10^4	5.88×10^4	7.34×10^4	8.21×10^4	0.03
109	KAF7080451.1	17,287	92	glycine cleavage system protein GcvH		1.66×10^5	3.12×10^4	1.98×10^4	3.45×10^4	4.79×10^4	1.79×10^4	2.31×10^4	3.56×10^4	3.25×10^4	5.19×10^4	3.14×10^4	5.14×10^4	0.04
110	KAF7061990.1	15,592	357	photosystem I reaction center subunit IV		1.69×10^5	9.70×10^4	1.22×10^5	1.43×10^5	6.19×10^3	6.86×10^3	8.34×10^3	9.16×10^3	2.95×10^4	4.48×10^4	1.62×10^4	9.58×10^4	0.03
111	ABB80135.1	26,160	198	vacuolar proton ATPase subunit	Ion transport	3.75×10^4	3.90×10^4	3.45×10^4	3.76×10^4	2.16×10^4	1.26×10^4	3.25×10^4	9.64×10^3	3.51×10^4	2.76×10^4	1.09×10^4	2.22×10^4	0.001
112	ACV89491.1	23,343	300	dehydroascorbate reductase	glutathione metabolism	3.42×10^4	4.40×10^4	3.84×10^4	1.78×10^4	1.80×10^5	2.65×10^5	1.52×10^5	1.98×10^5	1.33×10^4	2.21×10^4	3.78×10^5	3.26×10^5	0.03
113	CAC94001.1	24,984	132	glutathione transferase		5.22×10^4	1.50×10^4	2.04×10^4	1.15×10^4	1.85×10^4	1.14×10^4	2.05×10^4	2.16×10^4	2.18×10^4	1.15×10^4	1.24×10^4	1.05×10^4	0.04
114	AAA75104.1	16,245	486	single-stranded nucleic acid binding protein	RNA binding	9.93×10^4	1.77×10^4	1.33×10^4	1.94×10^4	4.71×10^5	3.37×10^5	4.22×10^5	3.33×10^5	1.60×10^4	1.95×10^4	3.13×10^5	3.49×10^5	0.02
115	AKQ09032.1	33,413	110	chitinase	carbohydrate metabolism	1.43×10^4	1.63×10^4	1.33×10^4	9.16×10^3	5.25×10^4	4.28×10^4	6.19×10^4	7.97×10^4	7.56×10^4	2.09×10^4	7.30×10^4	1.02×10^5	0.03
116	AAP70009.1	24,332	129	cytosolic malate dehydrogenase		4.41×10^4	8.14×10^4	8.81×10^4	7.54×10^4	7.72×10^3	1.34×10^4	8.40×10^3	1.87×10^4	5.47×10^4	1.24×10^5	1.95×10^4	2.69×10^4	0.04
117	ALE18234.1	36,586	394	glyceraldehyde-3-phosphate dehydrogenase	Glucose metabolic process	2.95×10^5	2.40×10^5	5.63×10^5	3.66×10^5	1.39×10^5	1.40×10^5	1.37×10^5	1.99×10^5	4.85×10^5	2.64×10^5	1.59×10^5	1.41×10^5	0.04
118	AVL25141.1	41,609	104	fructose-1,6-bisphosphate aldolase		1.08×10^4	2.51×10^4	1.18×10^4	6183	1.78×10^4	2.01×10^4	2.32×10^4	1.62×10^4	2.93×10^4	1.72×10^4	2.18×10^4	1.27×10^4	0.03
119	AEH16638.1	46,673	180	glutamine synthase	Nitrogen metabolism	1.09×10^5	1.00×10^5	2.23×10^5	9.28×10^4	4.96×10^4	7.22×10^4	3.97×10^4	7.90×10^4	8.23×10^4	1.14×10^5	1.05×10^5	8.99×10^4	0.04
120	AAP44537.1	25,875	301	cyclophilin-like protein	Protein folding	1.36×10^4	7.53×10^3	4.46×10^3	2.14×10^3	4.03×10^3	1.65×10^3	4.90×10^3	4.02×10^3	4.29×10^3	5.57×10^3	1.63×10^3	5.73×10^3	0.02
121	AAS17067.1	18,367	165	cyclophilin A		5.83×10^4	1.28×10^5	1.51×10^5	1.41×10^5	4.30×10^4	3.00×10^4	3.50×10^4	4.99×10^4	2.10×10^4	2.91×10^4	3.22×10^4	8.88×10^4	0.03
122	AIZ95472.1	16,047	148	actin depolymerizing factor 3	Actin binding	1.21×10^6	6.33×10^5	1.28×10^6	7.78×10^5	7.49×10^5	7.00×10^5	6.16×10^5	5.84×10^5	1.25×10^6	1.16×10^6	6.88×10^5	6.92×10^5	0.01
123	CAA52636.1	59,212	545	ATP synthase beta subunit	ATPase activity	3.50×10^4	7.62×10^4	8.60×10^4	1.25×10^5	1.28×10^5	1.23×10^5	1.20×10^5	1.37×10^5	5.98×10^4	1.04×10^5	1.51×10^5	8.25×10^4	0.02
124	AND74687.1	47,066	177	chloroplast ribulose biphosphate carboxylase/oxygenase activase		2.48×10^4	1.24×10^4	2.47×10^4	4.44×10^4	5.40×10^4	7.47×10^4	9.12×10^4	9.74×10^4	8.08×10^4	7.09×10^4	9.38×10^4	9.14×10^4	0.03
125	AGN94842.1	73,141	260	ER molecular charpeone		1.77×10^4	5.49×10^4	2.54×10^4	4.31×10^4	4.02×10^4	4.80×10^4	4.22×10^4	6.83×10^4	2.20×10^4	1.45×10^4	9.59×10^4	3.96×10^4	0.02
126	ABY85789.1	43,153	118	S-adenosylmethionine synthetase	ATP binding	2.87×10^4	6.98×10^4	3.62×10^4	8.69×10^4	1.48×10^4	3.34×10^4	2.08×10^4	2.16×10^4	1.05×10^5	5.97×10^4	5.56×10^4	2.48×10^4	0.04
127	KAF6989700.1	16,607	290	nucleoside diphosphate kinase		1.06×10^5	1.39×10^5	1.29×10^5	1.72×10^5	1.52×10^5	1.19×10^5	1.37×10^5	1.02×10^5	1.30×10^5	4.81×10^4	8.55×10^4	1.16×10^5	0.03
128	CAC85479.1	21,801	155	adenosine diphosphate glucose pyrophosphatase	Biosynthesis of starch	6.45×10^4	5.40×10^4	6.04×10^4	9.81×10^4	2.46×10^4	6.06×10^4	5.65×10^4	5.00×10^4	4.20×10^4	3.75×10^4	4.71×10^4	3.58×10^4	0.04

* Accession number—is a unique identifier given to a protein sequence record, identified by Mascot; ** Score—cumulative scores for individual peptides for all peptides matching a given protein; *** Spot volume—the sum of the pixel intensities within the spot area; **** *p* value between the highest and lowest normalized spot volumes.

The study of the proteomic profile revealed changes in important processes of individual systems. The root system is the main place of the microorganism–plant interaction and the main area affected by the herbicide.

The Table 1 shows results of the theoretical masses, accession number, scores, proteins ID obtained from the database search. The study of the results in the second part was based on selection of the data with the highest scores, comparison of their theoretical and experimental masses, definition of the protein function and its comparison to the earlier obtained results. It was found that some proteins differ in theoretical MW of from the proteins and mass obtained on the gel. It may be caused by several reasons: starting from the imperfections of gels due to the large numbers of treatment and trials, also the chemical modifications taking place in proteins may also have an impact on the changes [29].

According to UniProtKB specified functions, most proteins determined in our study were involved in stress response and antioxidation processes, such as oxidative stress response or superoxide metabolism, as well as in carbohydrate metabolism or glycolytic processes. Higher volumes of proteins and enzymes performing oxidoreductive functions, such as catalase, ascorbate peroxidase, cytochrome C peroxidase, and Cu/Zn superoxide dismutase, were found in the *Fusarium*-inoculated and 2,4-D-treated wheat roots. *Trichoderma* and its metabolites present in the system leveled out the mentioned proteins to the control volumes. These findings confirmed our earlier study on the enzyme activity levels.

Such enzymes as alpha amylase (theoretical MW 47,317 Da, SDS gel result ~46 kDa), malate dehydrogenase (theoretical MW 24,332 Da, in gel mass ~24 kDa), and S-adenosyl-methionine synthetase (theoretical MW 43,153 Da, analysis result ~44 kDa) function in carbohydrate metabolism, while cathepsin, having catalytic activity, dominates in *T. harzianum*-inoculated systems and cultures treated with its metabolites. Smaller volumes of these spots were specified in the samples treated with the herbicide or inoculated with the pathogen. However, chitinase (theoretical MW 28,242 Da, result in gel ~27 kDa)—which degrades polymer chitin into low-molecular-weight particles [30]—dominated in the presence of *F. culmorum*, which is in line with Boller's [31] findings that plant species may accumulate chitinases in response to plant pathogen infection. Due to this activity, chitinolytic enzymes have become some of the most promising candidates in the management of plant diseases [26].

An increased 12-oxo-phytodienoic acid reductase (theoretical MW 40,578 Da and in gel mas ~41 kDa) is involved in the oxylipin signaling pathway in wheat [32]. The activity of this protein was detected in herbicide-treated and *Fusarium*-inoculated wheat seeds. Additionally, there were significant changes in the oxylipin content in the roots of the analyzed samples. These results corroborate previously detected oxylipin levels.

Four proteins are deemed to be involved in the glycolytic process, one of which—glyceraldehyde-3-phosphate dehydrogenase (GAPDH) predominates in the entire proteomic profile. GAPDH catalyzes glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. This step is accompanied by the binding of phosphate to triosis in the metabolism of inorganic glucose [33]. In this study, the highest amount of this protein was found in control and *Trichoderma*-inoculated wheat systems. The smaller intensity of this spot in the wheat system in which *F. culmorum* was present was replaced with a higher value of triosephosphate isomerase (TPI) (theoretical MW 26,765 Da, in gel ~26 kDa). The understanding of the function of the TPI protein in plant–pathogen interactions is still very limited [34].

Another protein that shows clear differences in intensity and exhibits ATPase activity is ATP synthase beta (theoretical MW 59,212 Da, in gel ~60 kDa) The inhibition of this enzyme compromises the output of ATP by oxidative phosphorylation [35]. In our study, the intensity of this protein spot was lower in all *Fusarium*-inoculated systems.

Another large portion of proteins is involved in stress response and defense mechanisms, the value of which was shown to be predominant in the systems inoculated with the fungus. Chaperone proteins (theoretical MW 25,557 Da, in gel ~24 kDa) that assist the protein folding under stress conditions are essential for cell viability in all growth conditions [36]. Other germin-like proteins (GLPs) with plant response activity against abiotic

and biotic stresses include plant glycoproteins belonging to the cupin superfamily [37]. The advantage of these proteins is that they may function as antioxidant enzymes, which explains their higher content in all *F. culmorum*-inoculated and 2,4-D-treated samples.

Among the 30 studied proteins examined in the shoots, the proteins involved in photosynthesis and oxidative stress response attracted most of the attention. In this group, RUBISCO activase beta (theoretical MW 41,427 Da, in gel ~40 kDa), oxygen evolving enhancer (OEE) protein (theoretical MW 27,154 Da, in gel ~26 kDa), and ribulose 1,5 biphosphate arboxylase predominated in *T. harzianum*-inoculated systems and in wheat shoots grown with *Trichoderma* extracellular metabolites. The lowest intensity of the spots was found in the presence of *Fusarium* or 2,4-D. The OOE proteins are essential for photosynthesis and are involved in the photo-oxidation of water during the light reactions [38]. The absence of the protein results in reduced rates of photosynthetic oxygen evolution [39]. Zdražník et al. studied the drought stress effects on chloroplast proteins, with their findings describing the lower RWC linked to a lower amount of photosynthetic proteins [38]. Tracking our results, some dependence may be found between the lower RWC caused by chemical (2,4-D) or biological (*F. culmorum*) stress and the higher amount of photosynthetic proteins.

Another large portion of the studied proteins responsible for glutathione metabolism was observed and the amounts of these proteins were higher in the herbicide-treated sample and in *F. culmorum*-inoculated wheat. Plant glutathione transferase (theoretical MW 24,984 Da, in gel ~24 kDa), an enzyme capable of catalyzing the conjugation and detoxification of herbicides, was found in higher amounts in all 2,4-D-treated cultures in this study [40].

Dehydroascorbate reductase (DHAR) (theoretical MW 23,334 Da, in gel ~23 kDa), an enzyme belonging to the glutathione S-transferase (GST) superfamily, reduces glutathione in the ascorbate–glutathione pathway, which is believed to play a key role in H₂O₂ metabolism [41]. Substantial growth of this protein was found in all *Fusarium*-treated cultures. Even the presence of *Trichoderma* or its extract did not significantly lower the values. Parween et al. revealed that the application of exogenous JA under pesticide treatment induces the biosynthesis of ascorbate, glutathione, phenols, and tocopherol, which plays an important role in ROS scavenging [42]. According to our results, it can be seen that the increase in the amount of intracellular JA (as a response to pathogen infection) and the presence of the herbicide resulted in higher contents of proteins with oxidoreductive function.

Cyclophilins (theoretical MW 18,367 Da, in gel ~17 kDa) have been found to be versatile metabolites capable of regulating various processes in plant development and survival. They regulate multiple signaling and metabolic pathways and are able of binding such enzymes as thioredoxins and 2-Cys peroxiredoxin in chloroplasts [43]. In general, these proteins are present in different compartments of cells and are involved in various physiological processes. During our analysis, a higher content of these proteins was found in all systems with 2,4-D and in the *T. harzianum*-inoculated wheat system. Due to its ability for mRNA accumulation, S-adenosylmethionine synthetase (SAM) is an important protein of the defense mechanism in plants response to stress [44]. In our study, the largest spots of SAM were detected in all 2,4-D-treated cultures and *Trichoderma* metabolite-inoculated wheat seeds.

3. Materials and Methods

3.1. Reagents

All chemicals used in the proteomic analysis were purchased from Hercules, (Hercules, CA, USA), Promega (trypsin) (Madison, WI, USA), and Sigma-Aldrich (St. Louis, MO, USA). The oxylipin standards were obtained from Cayman Chemicals (Ann Arbor, MI, USA), while zearalenone and aurofusarin standards came from Sigma-Aldrich. The other materials, including solvents and plastic labware, were obtained from Avantor Performance Materials (Gliwice, Poland) and Eppendorf (Hamburg, Germany).

3.2. Plant Materials and Growth Examination

Triticum aestivum L. seeds (cv. Zyta) (Łódź, Poland) were selected based on the results of our previous research [5]. The culture was conducted as described in our previous work, with slight modifications. A total of 20 pre-sterilized seeds were placed onto two layers of filter paper and inserted onto 5 cm petri dishes. Next, 3 mL of water (with or without the herbicide and fungal spores) was added. During the germination, 1 mL of distilled water was added per day. The conditions of the plant growth chamber (IL/750/FIT P Pol-Eko, Poland) were not changed in a 14 h light/10 h dark photoperiod, with light supplied by cool white fluorescent lamps (a light intensity of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$) and the relative humidity being set to 40%.

The samples included one independent control with distilled water, 2,4-D-treated culture, *T. harzianum* alone and with 2,4-D, *F. culmorum* and *F. culmorum* with 2,4-D, *T. harzianum* with *F. culmorum*, and *F. culmorum* with 2,4-D. The extract of *T. harzianum* secondary metabolites was also studied alone and with 2,4-D, with *F. culmorum*, and separately with *F. culmorum* and 2,4-D. The 12 groups were examined and the process was repeated three times. The lengths of the shoots and roots were measured after 7 days of cultivation.

3.3. Fungal Inoculum

Trichoderma harzianum KKP534 from the fungal strain collection of the Department of Industrial Microbiology and Biotechnology, University of Lodz, and the strain *F. culmorum* DSM 1094 purchased from the German Collection of Microorganisms and Cell Cultures GmbH were used in the study. The strains were selected for the study based on our previous research [3], where *T. harzianum* extracellular metabolites caused the inhibition of *F. culmorum* growth and development.

Spores isolated from 7 days cultures grown on ZT or Sabouraud agar slants were used in the study [3]. The final concentration of 3×10^8 spores per Petri dish of each fungus were prepared for the experiment.

3.4. Trichoderma Extracts

Spores isolated from 7 days cultures grown on ZT agar slants (containing (in g/L): glucose, 4; Difco yeast extract, 4; agar, 25; malt extract, 6° Balling (BLG) up to 1 L (1° BLG = 1 g of soluble substances extracted from the grain per 100 mL of malt extract); pH 7.0) were used in the study.

The fungal spores inoculated in 20 mL Sabouraud dextrose broth medium (Difco) were added to 100 mL Erlenmeyer flasks [5]. The spores were cultured on a rotary shaker ($160 \times g$) for 48 h at 28 °C. Then, 2 mL of preculture was added to the Sabouraud medium. The cultures were incubated on a rotary shaker ($160 \times g$) at 28 °C. Following 24 h of incubation, the fungal cultures were filtered through a 115 mL filter unit (Thermo Scientific, Waltham, MA, USA), then 10 mL of the supernatant was transferred to a 50 mL Falcon tube.

The extracts were prepared according to the QuEChERS method described in our previous publication [3]. A 24 h *T. harzianum* culture was used for the study. After filtration, the supernatant was transferred into a 50 mL tube and 10 mL acetonitrile and the mix of salts (2 g MgSO_4 , 0.5 g NaCl , 0.5 g $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \times 2\text{H}_2\text{O}$, 0.25 g $\text{C}_6\text{H}_6\text{Na}_2\text{O}_7 \times 1.5\text{H}_2\text{O}$) were added. The tubes were shaken for 20 min using a rotary laboratory mixer. Subsequently, the tubes were centrifuged at $4000 \times g$ for 10 min at 4 °C, then the upper phase was transferred to new tubes and evaporated under pressure. Each extract was dissolved in 3 mL of solution, half of which was used for the one plant sample.

3.5. Relative Water Content

The relative water content (RWC) was determined on the 7th day of cultivation according to the Manzoni method [6]. The first component of the equation was the fresh plant shoot weight (W). According to the method used by Arndt et al. [45], the shoots were hydrated to full turgidity for 2 h, subsequently weighed, then the second parameter (TW)

was obtained. Then, the plant material was put into a laboratory oven at $-80\text{ }^{\circ}\text{C}$ for 24 h. When the shoots were completely dried and cooled, the last parameter (dry weight) was assessed (DW).

The RWC was determined according to the equation:

$$\text{RWC (\%)} = [(W - DW)/(TW - DW)] \times 100$$

The experiment was carried out three times and a total of 30 shoots were studied in each sample.

3.6. Chlorophyll Content Determination

The chlorophyll amount was determined after 7 days of cultivation. For this study, a Chlorophyll Content Meter CCM-300 (Opti-Sciences (Hudson, NY, USA)) was used. The measurement determined the CFR (the emission ratio of fluorescence) at wavelengths of 735 and 700 nm and the chlorophyll content is expressed in mg/m^2 based on the Gitelson equation. The measurements were carried out 4 times in three independent repetitions.

3.7. Metabolite Determination

Zearalenone was extracted from the filter paper. After 7 days of plant cultivation, the filter paper from Petri dishes was transferred to 50 mL tubes, then 5 mL of water and glass beads were added to each sample. The filter papers were crushed in a laboratory ball mill at a frequency of 30 min^{-1} for 3 min. Then, 10 mL of acetonitrile was added and the tubes were shaken again with the same parameters. The QuEChERS method (see Section 2.4) was performed with the obtained mixtures and the extracts were stored at $-20\text{ }^{\circ}\text{C}$ before analysis.

For zearalenone determination, the LC-MS/MS methods were applied. The extract was fractionated using the Agilent 1200 HPLC system (Agilent, Santa Clara, CA, USA). A Kinetex C18 column ($50\text{ mm} \times 2.1\text{ mm}$, particle size: $5\text{ }\mu\text{m}$; Phenomenex, (Torrance, CA, USA); column temperature $40\text{ }^{\circ}\text{C}$, injection volume $10\text{ }\mu\text{L}$) was applied for chromatographic separation. The eluents used in the study were water (A) and methanol (B), both containing 5 mM ammonium formate. The solvent was eluted at a constant flow rate of $500\text{ }\mu\text{L min}^{-1}$, starting with 80% of eluent A for 0.25 min, which then decreased to 10% of eluent A and was maintained for 4 min. The initial conditions were restored for a further 2 min. The MS/MS detection was performed using multiple reaction monitoring (MRM) mode during negative ionization. The monitored MRM pairs were $m/z\ 316.9 > 130.5$ and $316.9 > 174.1$ for zearalenone.

For *Trichoderma* metabolites, the same chromatographic steps were performed. The MS/MS detection was conducted using MRM mode during positive ionization [3].

3.8. CAT and SOD Activity

The enzymes activity study was performed according to Moura et al.'s method [46]. Briefly, 200 mg of fresh plant biomass (with shoots and roots separated) was ground in a mortar with freeze-drying in liquid nitrogen, then 1 mL of extraction buffer (0.05 M sodium phosphate buffer pH 7.8, 1 mM EDTA, 1%PVP) was added to the powdered tissue. The mixture was collected in the 1.5 mL tubes and centrifuged at $10,000 \times g$ at $4\text{ }^{\circ}\text{C}$ for 10 min. After the centrifugation, the supernatant was transferred into new tubes. The enzymes extracts were kept on ice.

The activity of catalase (CAT) was determined by measuring H_2O_2 degradation at $\lambda 240$. The superoxide dismutase (SOD) activity was measured by spectrophotometric evaluation of the inhibition of nitrotriazolium blue (NBT) chloride reduction at $\lambda 540$ [47].

3.9. Oxylin and Hormone Extraction and Determination

Oxylin were extracted from the roots or shoots according to the method used by Salem et al. with slight modifications [48]. Here, 100 mg of fresh plant tissue was ground in a mortar with freeze-drying in liquid nitrogen. The plant powder was transferred

into 2 mL tubes and 1 mL of MTBE/methanol solution (3:1) was added. The tubes were mixed in an orbital laboratory shaker for 30 min and centrifuged at $10,000\times g$ at $4\text{ }^{\circ}\text{C}$ for 10 min. The supernatant was partitioned to two new tubes, one of which was left at room temperature until dryness and transferred to $-20\text{ }^{\circ}\text{C}$ for further analysis. Before the oxylipin determination, the samples were defrosted and dissolved in methanol.

In the second tube with a half portion of the supernatant, 0.5 mL of 0.1% water HCl solution was added, then the tubes were shaken for 30 min in an orbital shaker for hormones analysis followed by centrifugation. The upper phase was dried at room temperature for 24 h and stored at $-20\text{ }^{\circ}\text{C}$ for further analysis. Before the hormone determination the samples were defrosted and dissolved in methanol.

Oxylipins were measured according to the method described in our previous work [3] using an Agilent 1200 HPLC system and a 4500 Q-TRAP mass spectrometer (Sciex, Foster City, CA, USA) with an ESI source.

3.10. Protein Extraction

Protein extraction from the plant tissue was based on the modified method used by Zhang et al. [49]. The previously prepared plant material was freeze-dried in liquid nitrogen and ground in a mortar until the tissue powder was obtained. A total of 0.25 g of the ground material was transferred into 2 mL low-binding Eppendorf tubes. Next, 1 mL of ice-cold 50% TCA/acetone with 1% β -mercaptoethanol was added, vortexed for 3 min, chilled on ice for 5 min, then centrifuged in a pre-cooled rotor at $13,500\times g$ at $4\text{ }^{\circ}\text{C}$ for 5 min. Then, the supernatant was removed and the last step was repeated until the samples were completely discolored. In further proceedings, the pellets were air-dried in the tubes at room temperature to remove the acetone residue. Next, 700 μL phenol with 0.5% DTT was added and the samples were incubated in an ultrasonic laboratory bath for 10 min, with the temperature kept below $30\text{ }^{\circ}\text{C}$. The centrifugation was performed according to the same parameters and the supernatant was placed in new tubes. The last step was repeated 3 times and the collected supernatant was transferred into new 2 mL tubes in an equal amount of 300 μL . For the precipitation step, methanol with 0.1% ammonium acetate was added into a volume of 5 mL, then an equivalent of the supernatant was supplemented and the tubes were incubated at $20\text{ }^{\circ}\text{C}$ overnight. After the precipitation, the samples were centrifuged in a pre-cooled rotor at $15,000\times g$ at $4\text{ }^{\circ}\text{C}$ for 20 min and the supernatant was removed.

For the purification step, the precipitates were collected in one tube within one sample and filled with 500 μL of guanidine hydrochloride (8M), then vortexed until the pellet dissolved. Next, tributyl phosphate (final concentration 5 mM) and 2-Vinyl-pyridin (final concentration 100 mM) were added. The samples were vortexed for 30 min in the dark and centrifuged as described earlier. The supernatant was transferred into two new tubes in equal amounts and 5 volumes of precooled acetone/methanol (1:1) were added. The precipitation was performed at $-20\text{ }^{\circ}\text{C}$ for 20 min and centrifugation at $13,500\times g$ was carried out at $4\text{ }^{\circ}\text{C}$ for 10 min. After the supernatant was removed, the pellets were collected in one tube within one sample, rinsed with methanol, vortexed for 3 min, and centrifuged on the same parameters as in the last step. This stage was performed three times. After the last centrifugation phase, the samples were dried in thermoblock and diluted in rehydration buffers (7 M urea, 2 M thiourea, 4% CHAPS, 0.01 M DTT). The samples prepared in this way were stored at $-70\text{ }^{\circ}\text{C}$ until analysis.

3.11. 2-D SDS PAGE

Two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (2D SDS PAGE) was conducted to separate the spots. Next, 300 μg of protein in 200 μL rehydration buffer was mixed with 7.5 μL of 40% ampholytes (pH range ~ 3.5 – 9.5) and 2 μL of bromophenol blue (1%). The full volume mix was loaded onto 11 cm IPG strips of pH 3–10 NL (non-linear) (cat no.163-2016, Bio-Rad, Germany) for overnight rehydration. Isoelectric focusing was performed using a Protean i12 device (Bio-Rad, Germany). The

assay was conducted according to the following parameters: 50 V for 4 h, linear gradient to 8000 V for 5 h, then held for 76,000 Vh. In the next step, the strips were equilibrated according to the Bio-Rad gel preparation protocol and the strips were placed on several polyacrylamide gels. As a gel calibrator, a 6500–200,000-Da molecular mass marker (Sigma-Aldrich) was used.

Same Spots software (the United Kingdom) was used to identify differences. The spots that differed in intensity were excised from the gels, digested, and used for further analyses.

3.12. Protein Identification

After excising the spots from the 2-DE gels manually, they were transferred to Eppendorf tubes (2.0 mL) for digestion as described by Szewczyk et al. [50]. Spectra were obtained using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF)/TOF (Sciex 5800 TOF/TOF system, Foster City, CA, USA) as described by Bernat et al. [51].

Protein Pilot v4.5 (Sciex) with the Mascot software v2.4 (Matrix Science, London, UK) was used for protein identification. The MS data were analyzed using the NCBI database with a taxonomy filter for *Triticum aestivum* (total number of sequences = 684,919).

To obtain the information on the functions of hypothetical proteins, the BLASTp algorithm in the nonredundant BLAST protein database was used. All searches were evaluated based on the significant scores obtained from MASCOT. The protein score was set to >95% and a significance threshold of $p < 0.05$ was used.

3.13. Statistical Analysis

In the germination study, growth condition parameters were measured and statistically analyzed for 20 seedlings, whereas all metabolomic experiments were carried out in three replicates. The standard error was determined and marked in the figures as an error bar. For more precise statistical analysis, the STATISTICA c.13.3 software was used. The study was performed using analysis of variance with Tukey's post hoc test. The data were considered as significant at $p < 0.05$. The results for all subcolumn analyses are attached as Supplementary Materials.

For the proteomic study, the proteins with scores greater than 68 (5% confidence threshold) were included as statistically significant.

4. Conclusions

This work was focused on the examination of how *T. harzianum* and its bioactive metabolites might influence germinated wheat defense against chemical stress (2,4-D) and biological infection (*F. culmorum*). The results indicated that the use of *Trichoderma* fungus or its extracellular metabolites could be an effective stimulant for plant development, as well as for the improvement of overall grain germination. The use of a living organism has proven to be a more effective method for minimizing oxidative stress in wheat caused by the interaction with *F. culmorum*. The obtained proteomics results confirmed the changes in the oxylipin profiles and the activity levels of CAT and SOD in the ROS scavenging mechanism. In the future, we plan to check the possibility of using *T. harzianum* for longer cultivation periods in order to trace further changes and to provide a more stable method for using metabolites.

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P-3
Supplementary material

Supplementary material

Table S1. Comparison of ANOVA Tukey post-hoc results for shoot and root lengths, with treatment as a factor, (T.h. – *Trichoderma harzianum*, F.c.- *Fusarium culmorum*, ex. T.h – *T. harzianum* extracellular metabolites treated wheat seeds), p significance was shown: * - $p \leq 0.001$, ns – not significant.

	ANOVA result for shoot length											
	Control	2,4D	T.h.	T.h.+2,4D	F.c.	F.c.+2,4D	T.h.+F.c.	T.h.+F.c.+2,4D	ex.T.h.	ex.T.h.+2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+2,4D
Control		*	*	*	*	*	ns	ns	*	*	*	*
2,4D	*		*	ns	ns	ns	*	*	*	ns	ns	ns
T.h.	*	*		*	*	*	ns	*	ns	*	*	*
T.h.+2,4D	*	ns	*		ns	ns	*	ns	*	ns	ns	ns
F.c.	*	ns	*	ns		ns	*	ns	*	ns	ns	ns
F.c.+2,4D	*	ns	*	ns	ns		*	*	*	ns	ns	ns
T.h.+F.c.	ns	*	ns	*	*	*		*	ns	*	*	*
T.h.+F.c.+2,4D	ns	*	*	ns	ns	*	*		*	ns	*	ns
ex.T.h.	*	*	ns	*	*	*	ns	*		*	*	*
ex.T.h.+2,4D	*	ns	*	ns	ns	ns	*	ns	*		ns	ns
ex.T.h.+F.c.	*	ns	*	ns	ns	ns	*	*	*	ns		ns
ex.T.h.+F.c.+2,4D	*	ns	*	ns	ns	ns	*	ns	*	ns	ns	
	ANOVA result for root length											
	2,4D	Control	T.h.	T.h.+2,4D	F.c.	F.c.+2,4D	T.h.+F.c.	T.h.+F.c.+2,4D	ex.T.h.	ex.T.h.+2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+2,4D
2,4D		*	*	ns	*	ns	*	ns	*	ns	*	ns
Control	*		*	*	*	*	ns	*	ns	*	*	*
T.h.	*	*		*	*	*	*	*	*	*	*	*
T.h.+2,4D	ns	*	*		*	ns	*	ns	*	ns	*	ns
F.c.	*	*	*	*		*	*	*	*	*	ns	*
F.c.+2,4D	ns	*	*	ns	*		*	ns	*	ns	*	ns
T.h.+F.c.	*	ns	*	*	*	*		*	ns	*	*	*
T.h.+F.c.+2,4D	ns	*	*	ns	*	ns	*		*	ns	*	ns
ex.T.h.	*	ns	*	*	*	*	ns	*		*	*	*
ex.T.h.+2,4D	ns	*	*	ns	*	ns	*	ns	*		*	ns
ex.T.h.+F.c.	*	*	*	*	ns	*	*	*	*	*		*
ex.T.h.+F.c.+2,4D	ns	*	*	ns	*	ns	*	ns	*	ns	*	

Table S2. Comparison of ANOVA Tukey post-hoc results for seeds germination with treatment as a factor, (T.h. – *Trichoderma harzianum*, F.c.- *Fusarium culmorum*, ex. T.h – *T. harzianum* extracellular metabolites treated wheat seeds), p significance was shown: * - $p \leq 0.001$, ns – not significant.

	ANOVA result for germination											
	Control	2,4D	T.h.	T.h.+2,4D	F.c.	F.c.+2,4D	T.h.+F.c.	T.h.+F.c.+2,4D	ex.T.h.	ex.T.h.+2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+2,4D
Control		ns	ns	ns	*	*	*	*	ns	ns	*	*
2,4D	ns		ns	ns	*	*	*	*	ns	ns	*	*
T.h.	ns	ns		ns	*	*	*	*	ns	ns	*	*
T.h.+2,4D	ns	ns	ns		*	*	*	*	ns	ns	*	*
F.c.	*	*	*	*		ns	ns	ns	*	*	ns	ns
F.c.+2,4D	*	*	*	*	ns		ns	ns	*	*	ns	ns
T.h.+F.c.	*	*	*	*	ns	ns		ns	*	*	ns	ns
T.h.+F.c.+2,4D	*	*	*	*	ns	ns	ns		*	*	ns	ns
ex.T.h.	ns	ns	ns	ns	*	*	*	*		ns	*	*
ex.T.h.+2,4D	ns	ns	ns	ns	*	*	*	*	ns		*	*
ex.T.h.+F.c.	*	*	*	*	ns	ns	ns	ns	*	*		ns
ex.T.h.+F.c.+2,4D	*	*	*	*	ns	ns	ns	ns	*	*	ns	

Table S3. Comparison of ANOVA Tukey post-hoc results for RWC with treatment as a factor, (T.h. – *Trichoderma harzianum*, F.c.- *Fusarium culmorum*, ex. T.h – *T. harzianum* extracellular metabolites treated wheat seeds), p significance was shown* $p \leq 0.001$, ** $p \leq 0.0001$, ns – not significant.

	ANOVA result for RWC											
	Control	2,4D	T.h.	T.h.+2,4D	F.c.	F.c.+2,4D	T.h.+F.c.	T.h.+F.c.+2,4D	ex.T.h.	ex.T.h.+2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+2,4D
Control		*	ns	ns	**	**	**	**	ns	*	**	**
2,4D	*		ns	ns	**	**	ns	**	*	ns	ns	*
T.h.	ns	ns		ns	**	**	**	**	ns	ns	*	**
T.h.+2,4D	ns	ns	ns		**	**	**	**	ns	ns	ns	**
F.c.	**	**	**	**		ns	**	ns	**	**	**	**
F.c.+2,4D	**	**	**	**	ns		**	ns	**	**	**	**
T.h.+F.c.	**	ns	**	**	**	**		**	**	ns	ns	ns
T.h.+F.c.+2,4D	**	**	**	**	ns	ns	**		**	**	**	**
ex.T.h.	ns	*	ns	ns	**	**	**	**		ns	*	**
ex.T.h.+2,4D	*	ns	ns	ns	**	**	ns	**	ns		ns	*
ex.T.h.+F.c.	**	ns	*	ns	**	**	ns	**	*	ns		ns
ex.T.h.+F.c.+2,4D	**	*	**	**	**	**	ns	**	**	*	ns	

Table S4. Comparison of ANOVA Tukey post-hoc results for chlorophyll content with treatment as a factor, (T.h. – *Trichoderma harzianum*, F.c.- *Fusarium culmorum*, ex. T.h – *T. harzianum* extracellular metabolites treated wheat seeds), p significance was shown: * $p \leq 0.001$, ** $p \leq 0.0001$, ns – not significant.

[illegible]

Table S5. Comparison of ANOVA Tukey post-hoc results for JA with treatment as a factor, (T.h. – *Trichoderma harzianum*, F.c.- *Fusarium culmorum*, ex. T.h – *T. harzianum* extracellular metabolites treated wheat seeds), p significance was shown: ** - $p \leq 0.001$, * - $p \leq 0.01$, ns – not significant.

	ANOVA result for JA											
	Control	2,4D	T.h.	T.h.+2,4D	F.c.	F.c.+2,4D	T.h.+F.c.	T.h.+F.c.+2,4D	ex.T.h.	ex.T.h.+2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+2,4D
Control		**	ns	**	**	**	**	**	*	**	**	**
2,4D	**		**	*	**	**	**	*	**	**	**	**
T.h.	ns	**		**	**	**	**	**	**	**	**	**
T.h.+2,4D	**	*	**		**	**	**	ns	**	**	**	ns
F.c.	**	**	**	**		**	ns	**	**	**	**	**
F.c.+2,4D	**	**	**	**	**		**	**	**	ns	*	**
T.h.+F.c.	**	**	**	**	ns	**		**	**	**	**	**
T.h.+F.c.+2,4D	**	*	**	ns	**	**	**		**	**	**	*
ex.T.h.	*	**	*	**	**	**	**	**		**	**	**
ex.T.h.+2,4D	**	**	**	**	**	ns	**	**	**		**	**
ex.T.h.+F.c.	**	ns	**	**	**	*	**	**	**	**		ns
ex.T.h.+F.c.+2,4D	**	ns	**	ns	**	**	**	*	**	**	ns	

Table S6. Comparison of ANOVA Tukey post-hoc results for oxylipin with treatment as a factor, (T.h. – *Trichoderma harzianum*, F.c.- *Fusarium culmorum*, ex. T.h – *T. harzianum* extracellular metabolites treated wheat seeds), p significance was shown: ** - $p \leq 0.001$, * - $p \leq 0.01$, ns – not significant

	9-Hotre (shoot)											
	Control	2,4D	T.h.	T.h.+2,4D	F.c.	F.c.+2,4D	T.h.+F.c.	T.h.+F.c.+2,4D	ex.T.h.	ex.T.h.+2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+2,4D
Control		*	*	*	*	ns	*	*	*	*	*	*
2,4D	*		*	ns	*	*	ns	*	*	*	*	*
T.h.	*	*		*	*	*	*	*	ns	*	*	*
T.h.+2,4D	*	ns	*		*	*	ns	*	*	*	*	*
F.c.	*	*	*	*		*	ns	*	*	ns	*	*
F.c.+2,4D	ns	*	*	*	*		*	*	*	*	*	*
T.h.+F.c.	*	ns	*	ns	ns	*		*	*	*	*	*
T.h.+F.c.+2,4D	*	*	*	*	*	*	*		ns	*	*	*
ex.T.h.	*	*	ns	*	*	*	*	ns		*	*	*
ex.T.h.+2,4D	*	*	*	*	ns	*	*	*	*		*	*
ex.T.h.+F.c.	*	*	*	*	*	*	*	*	*	*		ns
ex.T.h.+F.c.+2,4D	*	*	*	*	*	*	*	*	*	*	ns	
	13-HODE (shoot)											
	Control	2,4D	T.h.	T.h.+2,4D	F.c.	F.c.+2,4D	T.h.+F.c.	T.h.+F.c.+2,4D	ex.T.h.	ex.T.h.+2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+2,4D
Control		*	*	*	*	ns	ns	*	*	ns	*	*
2,4D	*		*	*	*	*	*	*	*	*	*	ns
T.h.	*	*		ns	*	*	*	*	ns	*	*	*
T.h.+2,4D	*	*	ns		*	*	*	ns	ns	*	*	*
F.c.	*	*	*	*		*	*	*	*	*	*	*
F.c.+2,4D	ns	*	*	*	*		ns	*	*	ns	ns	*
T.h.+F.c.	ns	*	*	*	*	ns		*	*	ns	ns	*
T.h.+F.c.+2,4D	*	*	*	ns	*	*	*		ns	*	*	*
ex.T.h.	*	*	ns	ns	*	*	*	ns		*	*	*
ex.T.h.+2,4D	ns	*	*	*	*	ns	ns	*	*		ns	*
ex.T.h.+F.c.	*	*	*	*	*	ns	ns	*	*	ns		*

ex.T.h.+F.c.+2,4 D	*	ns	*	*	*	*	*	*	*	*	*	*
	9-HODE (shoot)											
	Control	2,4D	T.h.	T.h.+2,4D	F.c.	F.c.+2,4D	T.h.+F.c.	T.h.+F.c.+2,4D	ex.T.h.	ex.T.h.+2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+2,4 D
Control		*	*	*	*	*	*	*	*	*	*	ns
2,4D	*		*	*	*	*	*	*	*	*	*	*
T.h.	*	*		ns	*	*	*	ns	ns	*	ns	*
T.h.+2,4D	*	*	ns		*	ns	*	ns	ns	*	ns	*
F.c.	*	*	*	*		*	*	*	*	*	*	*
F.c.+2,4D	*	*	*	ns	*		*	ns	ns	ns	ns	*
T.h.+F.c.	*	*	*	*	*	*		*	*	ns	*	*
T.h.+F.c.+2,4D	*	*	ns	ns	*	ns	*		ns	*	ns	*
ex.T.h.	*	*	ns	ns	*	ns	*	ns		*	ns	*
ex.T.h.+2,4D	*	*	*	*	*	ns	ns	*	*		*	*
ex.T.h.+F.c.	*	*	ns	ns	*	ns	*	ns	ns	*		*
ex.T.h.+F.c.+2,4 D	ns	*	*	*	*	*	*	*	*	*	*	
	13-Hotre (shoot)											
	Control	2,4D	T.h.	T.h.+2,4D	F.c.	F.c.+2,4D	T.h.+F.c.	T.h.+F.c.+2,4D	ex.T.h.	ex.T.h.+2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+2,4 D
Control		ns	*	ns	*	ns	*	*	*	*	*	ns
2,4D	ns		*	ns	*	ns	*	*	*	*	*	ns
T.h.	*	*		*	*	ns	ns	ns	*	ns	ns	*
T.h.+2,4D	ns	ns	*		*	ns	*	*	*	*	*	ns
F.c.	*	*	*	*		*	*	*	*	*	*	*
F.c.+2,4D	ns	ns	ns	ns	*		*	*	*	*	*	ns
T.h.+F.c.	*	*	ns	*	*	*		ns	ns	ns	ns	*
T.h.+F.c.+2,4D	*	*	ns	*	*	*	ns		*	ns	ns	*
ex.T.h.	*	*	*	*	*	*	ns	*		ns	ns	*
ex.T.h.+2,4D	*	*	ns	*	*	*	ns	ns	ns		ns	*
ex.T.h.+F.c.	*	*	ns	*	*	*	ns	ns	ns	ns		*
ex.T.h.+F.c.+2,4 D	ns	ns	*	ns	*	ns	*	*	*	*	*	

9Hotre (root)												
	Control	2,4D	T.h.	T.h.+2,4D	F.c.	F.c.+2,4D	T.h.+F.c.	T.h.+F.c.+2,4D	ex.T.h.	ex.T.h.+2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+2,4 D
Control		*	*	ns	*	ns	*	*	*	*	*	*
2,4D	*		*	ns	*	*	*	ns	*	*	*	ns
T.h.	*	*		*	*	*	*	*	*	*	*	*
T.h.+2,4D	ns	ns	*		*	ns	*	ns	*	*	*	ns
F.c.	*	*	*	*		*	*	*	ns	*	*	*
F.c.+2,4D	ns	*	*	ns	*		*	*	*	*	*	*
T.h.+F.c.	*	*	*	*	*	*		*	*	*	*	*
T.h.+F.c.+2,4D	*	ns	*	ns	*	*	*		*	*	*	ns
ex.T.h.	*	*	*	*	ns	*	*	*		*	*	*
ex.T.h.+2,4D	*	*	*	*	*	*	*	*	*		ns	ns
ex.T.h.+F.c.	*	*	*	*	*	*	*	*	*	ns		*
ex.T.h.+F.c.+2,4 D	*	ns	*	ns	*	*	*	ns	*	ns	*	

13Hode (root)												
	Control	2,4D	T.h.	T.h.+2,4D	F.c.	F.c.+2,4D	T.h.+F.c.	T.h.+F.c.+2,4D	ex.T.h.	ex.T.h.+2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+2,4 D
Control		*	*	*	*	ns	*	*	*	ns	*	*
2,4D	*		*	*	ns	*	*	ns	*	*	*	*
T.h.	*	*		*	*	*	*	*	*	*	*	*
T.h.+2,4D	*	*	*		*	*	*	*	*	*	ns	ns
F.c.	*	ns	*	*		*	*	ns	*	ns	*	*
F.c.+2,4D	ns	*	*	*	*		*	*	*	ns	*	*
T.h.+F.c.	*	*	*	*	*	*		*	ns	*	*	*
T.h.+F.c.+2,4D	*	ns	*	*	ns	*	*		*	ns	*	*
ex.T.h.	*	*	*	*	*	*	ns	*		*	*	*
ex.T.h.+2,4D	ns	*	*	*	ns	ns	*	ns	*		*	*
ex.T.h.+F.c.	*	*	*	ns	*	*	*	*	*	*		*
ex.T.h.+F.c.+2,4 D	*	*	*	ns	*	*	*	*	*	*	*	

9Hode (root)

Table S7. Comparison of ANOVA Tukey post-hoc results for CAT and SOD with treatment as a factor, (T.h. – *Trichoderma harzianum*, F.c.- *Fusarium culmorum*, ex. T.h – *T. harzianum* extracellular metabolites treated wheat seeds), p significance was shown: * - $p \leq 0.05$ ** - $p \leq 0.01$, *** - $p \leq 0.001$, ns – not significant.

	ANOVA for CAT (shoot)											
	Control	2,4-D	T.h	T.h 2,4-D	F.c.	F.c. 2,4-D	T.h. F.c.	T.h. F.c. 2,4-D	e.T.h.	e.T.h. 2,4-D	e.T.h. F.c.	e.T.h. F.c. 2,4-D
Control		**	ns	*	***	***	***	***	***	***	***	***
2,4-D	**		ns	ns	***	***	ns	***	ns	ns	ns	***
T.h	ns	ns		ns	***	***	***	***	**	**	*	***
T.h 2,4-D	*	ns	ns		***	***	***	***	**	**	ns	***
F.c.	***	***	***	***		ns	ns	***	**	*	**	ns
F.c. 2,4-D	***	***	***	***	ns		***	*	***	***	***	ns
T.h. F.c.	***	ns	***	***	ns	***		***	ns	ns	ns	***
T.h. F.c. 2,4-D	***	***	***	***	***	*	***		***	***	***	ns
e.T.h.	***	ns	**	**	**	***	ns	***		ns	ns	***
e.T.h. 2,4-D	***	ns	**	**	*	***	ns	***	ns		ns	***
e.T.h. F.c.	***	ns	*	ns	**	***	ns	***	ns	ns		***
e.T.h. F.c. 2,4-D	***	***	***	***	ns	ns	***	ns	***	***	***	

	ANOVA for CAT (root)											
	Control	2,4-D	T.h	T.h 2,4-D	F.c.	F.c. 2,4-D	T.h. F.c.	T.h. F.c. 2,4-D	e.T.h.	e.T.h. 2,4-D	e.T.h. F.c.	e.T.h. F.c. 2,4-D
Control		ns	ns	ns	ns	***	ns	***	ns	*	ns	ns
2,4-D	ns		ns	ns	ns	***	ns	**	ns	ns	ns	ns
T.h	ns	ns		ns	ns	***	ns	**	ns	ns	ns	ns
T.h 2,4-D	ns	ns	ns		ns	***	ns	***	ns	*	ns	ns
F.c.	ns	ns	ns	ns		***	ns	*	ns	ns	ns	ns
F.c. 2,4-D	***	***	***	***	***		***	ns	**	*	***	***
T.h. F.c.	ns	ns	ns	ns	ns	***		***	ns	**	ns	*
T.h. F.c. 2,4-D	***	**	**	***	*	ns	***		**	ns	***	ns

e.T.h.	ns	ns	ns	ns	ns	***	ns	**		ns	ns	ns
e.T.h. 2,4-D	*	ns	ns	*	ns	*	**	ns	ns		ns	ns
e.T.h. F.c.	ns	ns	ns	ns	ns	***	ns	**	ns	ns		ns
e.T.h. F.c. 2,4-D	ns	ns	ns	ns	ns	**	*	ns	ns	ns	ns	

ANOVA for SOD (shoot)												
	Control	2,4-D	T.h	T.h 2,4-D	F.c.	F.c. 2,4-D	T.h. F.c.	T.h. F.c. 2,4-D	e.T.h.	e.T.h. 2,4-D	e.T.h. F.c.	e.T.h. F.c. 2,4-D
Control		ns	ns	ns	ns	***	ns	ns	ns	ns	ns	***
2,4-D	ns		ns	ns	ns	***	*	ns	ns	ns	ns	***
T.h	ns	ns		ns	ns	***	ns	ns	*	ns	ns	***
T.h 2,4-D	ns	ns	ns		ns	***	ns	ns	*	ns	ns	***
F.c.	ns	ns	ns	ns		***	ns	ns	ns	ns	ns	***
F.c. 2,4-D	***	***	***	***	***		***	***	**	***	***	ns
T.h. F.c.	ns	*	ns	ns	ns	***		ns	**	ns	ns	***
T.h. F.c. 2,4-D	ns	ns	ns	ns	ns	***	ns		ns	ns	ns	***
e.T.h.	ns	ns	*	ns	ns	**	**	ns		ns	ns	***
e.T.h. 2,4-D	ns	ns	ns	ns	ns	***	ns	ns	ns		ns	***
e.T.h. F.c.	ns	ns	ns	ns	ns	***	ns	ns	ns	ns		***
e.T.h. F.c. 2,4-D	***	***	***	***	***	ns	***	***	***	***	***	

ANOVA for SOD (root)												
	Control	2,4-D	T.h	T.h 2,4-D	F.c.	F.c. 2,4-D	T.h. F.c.	T.h. F.c. 2,4-D	e.T.h.	e.T.h. 2,4-D	e.T.h. F.c.	e.T.h. F.c. 2,4-D
Control		ns	***	**	***	***	***	ns	ns	**	***	ns
2,4-D	ns		ns	ns	***	***	***	***	ns	ns	***	***
T.h	***	ns		ns	***	***	***	***	ns	ns	***	***
T.h 2,4-D	**	ns	ns		***	***	***	***	ns	ns	***	***
F.c.	***	***	***	***		ns	ns	***	***	***	***	***
F.c. 2,4-D	***	***	***	***	ns		***	***	***	***	***	***
T.h. F.c.	***	***	***	***	ns	***		***	***	***	***	***
T.h. F.c. 2,4-D	ns	*	***	***	***	***	***		***	***	***	ns
e.T.h.	ns	ns	ns	ns	***	***	***	***		ns	***	***
e.T.h. 2,4-D	**	ns	ns	ns	***	***	***	***	ns		***	***
e.T.h. F.c.	***	***	***	***	***	***	***	***	***	***		*
e.T.h. F.c. 2,4-D	ns	***	***	***	***	***	***	ns	***	***	*	

Table S8. Comparison of ANOVA Tukey post-hoc results for ZEA with treatment as a factor, (T.h. – *Trichoderma harzianum*, F.c.- *Fusarium culmorum*, ex. T.h – *T. harzianum* extracellular metabolites treated wheat seeds), p significance was shown: * $p \leq 0.01$, ** $p \leq 0.001$, ns – not significant

	ANOVA for ZEA					
	F.c.	F.c.+2,4D	T.h.+F.c.	T.h.+F.c.+2,4D	ex.T.h.+F.c.	ex.T.h.+F.c.+2,4D
F.c.		**	*	ns	**	ns
F.c.+2,4D	**		**	**	**	*
T.h.+F.c.	*	**		ns	**	ns
T.h.+F.c.+2,4D	ns	**	ns		**	ns
ex.T.h.+F.c.	**	**	**	**		**
ex.T.h.+F.c.+2,4D	ns	*	ns	ns	**	

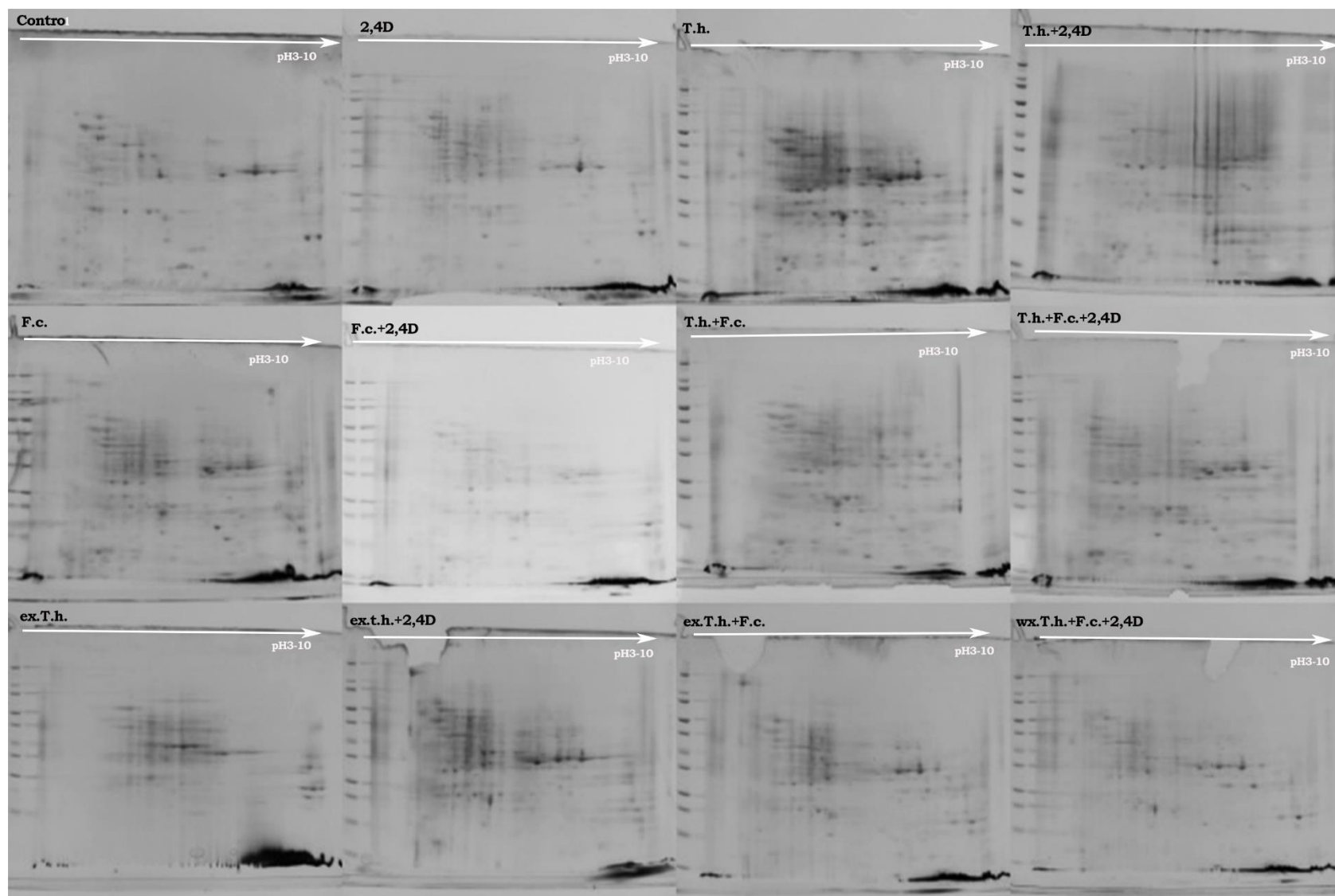


Figure S1. 2-D SDS PAGE protein profile from root material (Control; 2,4D; *T. harzianum* inoculated; *T. harzianum* + 2,4D; *F. culmorum* inoculated; *F. culmorum* + 2,4D; *T. harzianum* + *F. culmorum*; *T. harzianum* + *F. culmorum* + 2,4D; treated with extracellular extract *T. harzianum*; with extracellular extract *T. harzianum* + 2,4D; with extracellular extract *T. harzianum* + *F. culmorum*; extracellular extract *T. harzianum* + *F. culmorum* + 2,4D).

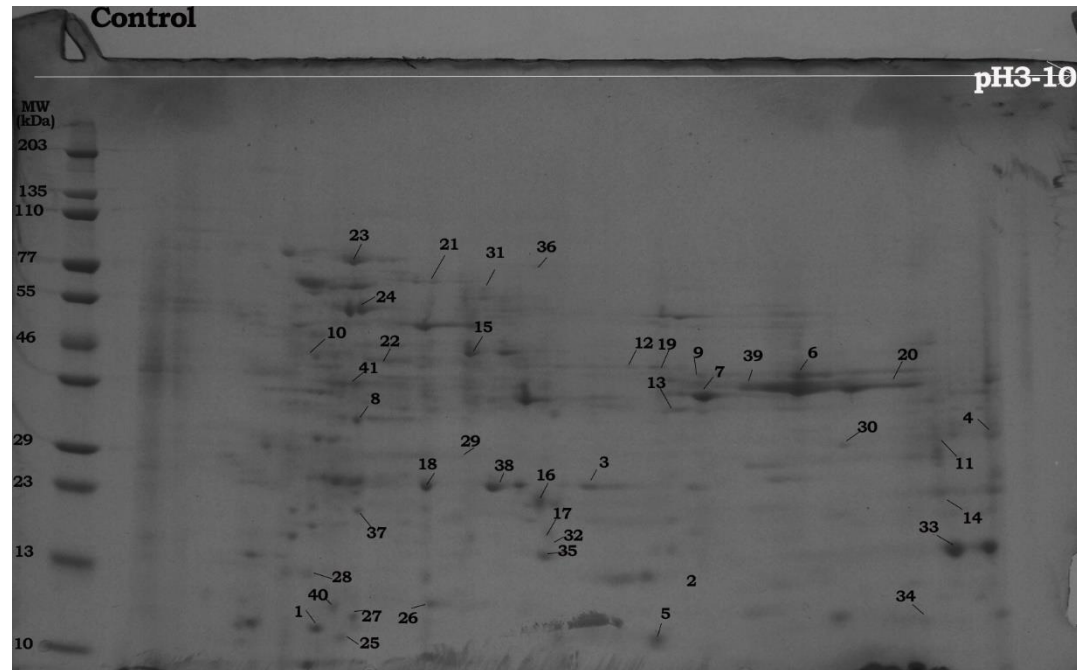


Figure S2. The spots analyzed during the proteomic study from root material, applied to a control gel, as an example.

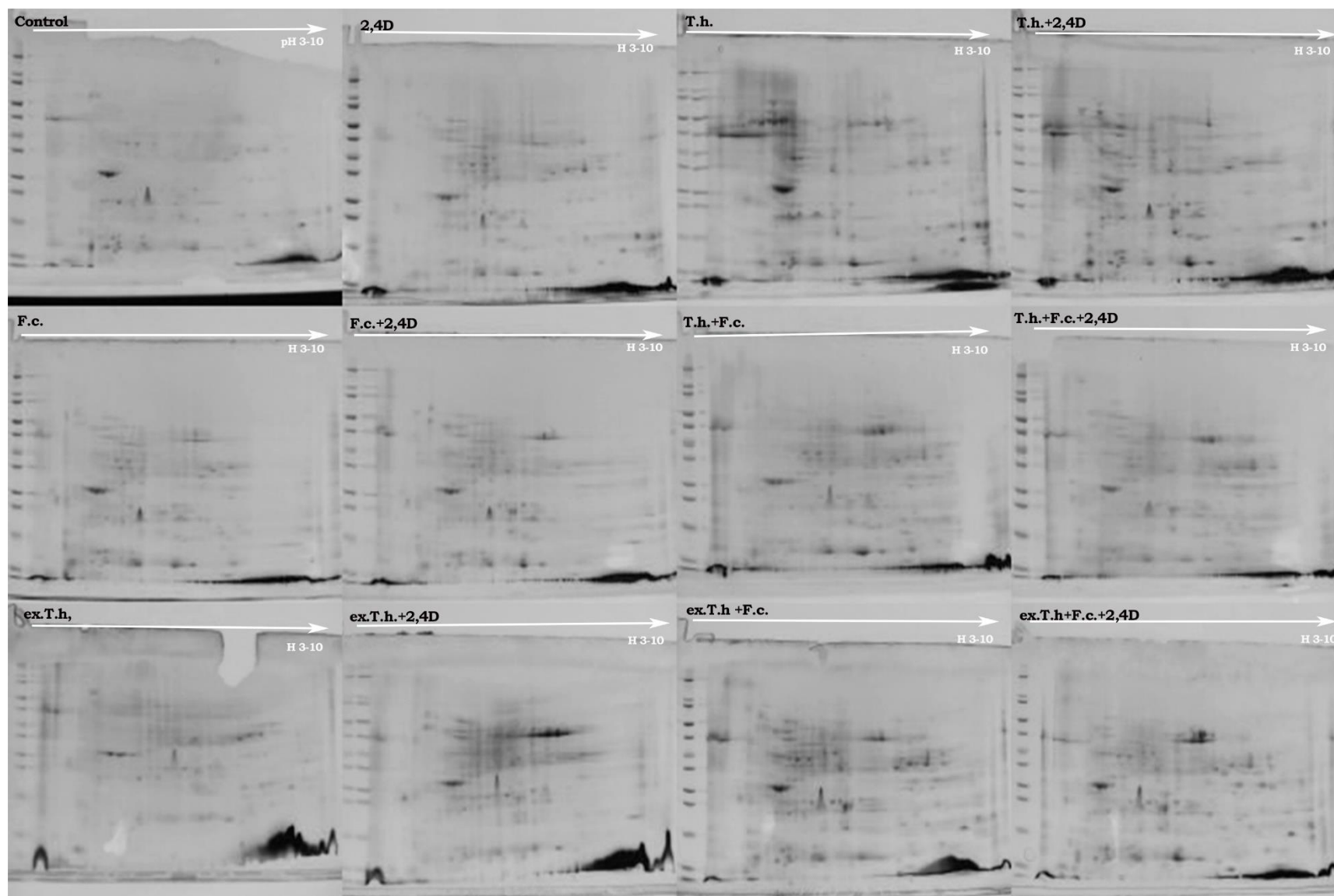


Figure S3. 2-D SDS PAGE protein profile from shoot material (Control; 2,4D; *T. harzianum* inoculated; *T. harzianum* + 2,4D; *F. culmorum* inoculated; *F. culmorum* + 2,4D; *T. harzianum* + *F. culmorum*; *T. harzianum* + *F. culmorum* + 2,4D; treated with extracellular extract *T. harzianum*; with extracellular extract *T. harzianum* + 2,4D; with extracellular extract *T. harzianum* + *F. culmorum*; extracellular extract *T. harzianum* + *F. culmorum* + 2,4D).

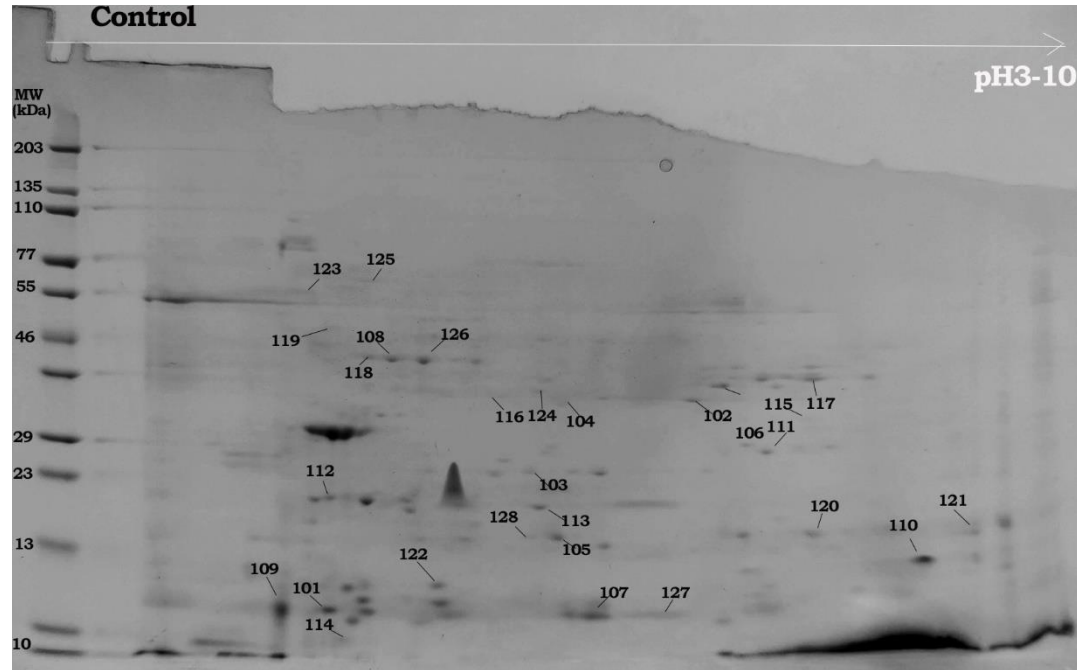


Figure S4. The spots analyzed during the proteomic study from shoot material, applied to a control gel, as an example.

10. Podsumowanie i stwierdzenia końcowe

- *T. harzianum* nie degraduje 2,4-D.
- Zwiększona płynność i przepuszczalność błon oraz modyfikacje w profilu fosfolipidów i sfingolipidów grzybowych to możliwe odpowiedzi adaptacyjne drobnoustroju na obecność herbicydu w hodowli.
- Dodatek do hodowli 2,4-D wpływa negatywnie na proces syntezy zewnątrzkomórkowych metabolitów *T. harzianum*, związków o właściwościach grzybobójczych i wspomagających wzrost roślin.
- Ekspresja zewnątrzkomórkowych białek stresowych wskazywała na toksyczne działanie dodanego do hodowli herbicydu na drobnoustrój.
- Obecność 2,4-D w hodowli płynnej indukuje nadprodukcję RFT w grzybnii *T. harzianum*.
- Metabolity obecne w płynie pohodowlanym *T. harzianum* negatywnie wpływają na wzrost i zdolność do zarodnikowania *F. culmorum* w hodowli na podłożu stałym.
- Różnice w wytwarzaniu barwników naftochinonowych oraz zearalenonu wskazują na wpływ dodanych metabolitów na prawidłowy rozwój drobnoustroju.
- W hodowli płynnej dodanie ekstraktu indukuje produkcję RFT i prowadzi do ekspresji białek o właściwościach oksydoredukcyjnych wybranego do badań modelu *Fusarium*.
- Zaszczepienie ziaren pszenicy zarodnikami *F. culmorum* zmniejsza zdolność wykiełkowania o ponad 80%.
- W obecności 2,4-D następuje spowolnienie tempa kiełkowania ziaren pszenicy i rozwoju systemu korzeniowego rośliny
- Zaszczepienie ziaren pszenicy *T. harzianum* przyspiesza wzrost i kiełkowanie roślin, a w układach z biotycznym czynnikiem stresowym, obserwowane są metabolity oddziałujące negatywnie na zarodniki *F. culmorum*. W układzie z dodanym herbicydem, *T. harzianum* wspomaga kiełkowanie pszenicy niwelując toksyczne działanie 2,4-D.
- Zaaplikowanie ekstraktów pochodzących z płynu pohodowlanego *T. harzianum* miało porównywalny wpływ na wzrost i kiełkowanie pszenicy w

stosunku do układów z zarodnikami tego grzyba. W układach z dodanym czynnikiem stresu abiotycznego zaaplikowany ekstrakt nie był tak skuteczny jak dodanie zarodników grzybowych, natomiast w układach z fitopatogenem we wczesnych stadiach wykazywał porównywalną skuteczność.

11. Potencjalne zastosowanie praktyczne

Przeprowadzone badania w pracy doktorskiej przybliżyły możliwość zagospodarowania płynu pochodzącego z *T. harzianum* jak i zarodników drobnoustroju, w celu ochrony pszenicy przed czynnikami stresowymi biotycznymi i abiotycznymi. Oceniono w warunkach laboratoryjnych zdolności *T. harzianum* do ulepszenia parametrów wzrostu pszenicy w obecności 2,4-D i *F. culmorum*, które można przełożyć po przeprowadzeniu reoptymalizacji na uprawy, w celu dalszych badań z możliwością wprowadzenia do użytku agroprzemysłowego.

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13. Oświadczenie współautorów o udziale w publikacjach

Oświadczenie współautorów o udziale w publikacji

Mironenka J., Różalska S., Soboń A., Bernat P. (2020) „Lipids, proteins and extracellular metabolites of *Trichoderma harzianum* modifications caused by 2,4-dichlorophenoxyacetic acid as a plant growth stimulator”. *Ecotoxicology and Environmental Safety*, vol. 194, 1-10. (IF: 6,23; MNiSW: 100).

Imię i Nazwisko	Szacunkowy udział [%]	Opis działań
mgr Julia Mironenka	65	Opracowanie koncepcji badań obejmujących wpływ 2,4D na syntezę metabolitów wtórnych przez <i>Trichoderma harzianum</i> . Zaplanowanie i realizacja doświadczeń dotyczących oceny zdolności do wzrostu oraz produkcji metabolitów wtórnych. Analiza zmian w przepuszczalności błony komórkowej badanego grzyba, oznaczenia profilu lipidowego. Ocena wpływu herbicydu na zewnątrzkomórkowy proteom drobnoustroju. Statystyczna analiza otrzymanych wyników. Przygotowanie podstawowej wersji manuskryptu, opracowanie graficzne wyników. Udział w przygotowaniu odpowiedzi dla recenzentów. Julia Mironenka (podpis współautora)
dr hab. Sylwia Różalska, prof. UŁ	10	Współudział w konsultacjach metodycznych, przeprowadzenie, analiza i interpretacja badań dotyczących oznaczeń wpływu 2,4D na zmiany w zawartości anionorodników w hodowli <i>T. harzianum</i> , z użyciem techniki mikroskopii konfokalnej oraz analiza i interpretacja uzyskanych wyników. Różalska (podpis współautora)
dr Adrian Soboń	10	Współudział w opracowaniu metodyki i wykonaniu doświadczeń profilu białek zewnątrzkomórkowych, a także udział w edycji opracowanych wyników w manuskrypcie. Soboń (podpis współautora)
dr hab. Przemysław Bernat, prof. UŁ	15	Współudział w koncepcji pracy; projektowanie metod, przeprowadzenie badań, ocena postępów, konsultacje metodyczne oraz uczestnictwo w przygotowaniu manuskryptu, a także edycji pracy, autor korespondujący. Bernat (podpis współautora)

Oświadczenie współautorów o udziale w publikacji

Mironenka J., Różalska S., Soboń A., Bernat P. (2021) “*Trichoderma harzianum* metabolites disturb *Fusarium culmorum* metabolism: Metabolomic and proteomic studies”.

Microbiological Research, vol.249, 126770. (IF: 5,415; MNiSW: 100)

Imię i Nazwisko	Szacunkowy udział [%]	Opis działań
mgr Julia Mironenka	70	Opracowanie koncepcji badań obejmujących wpływ metabolitów <i>T. harzianum</i> na wzrost i produkcję mykotoksyn <i>Fusarium culmorum</i> . Zaplanowanie i realizacja doświadczeń dotyczących hodowli na podłożu stałym i w hodowli płynnej. Analiza zdolności wytwarzania mykotoksyn. Porównanie wpływu dodanych metabolitów na wewnątrzkomórkowy proteom wybranego grzyba. Statystyczna analiza otrzymanych wyników. Przygotowanie manuskryptu, opracowanie graficzne wyników. Udział w przygotowaniu odpowiedzi dla recenzentów. <i>Julia Mironenka</i> (podpis współautora)
dr hab. Sylwia Różalska, prof. UŁ	10	Współudział w konsultacjach metodycznych, analizie wyników, opracowania manuskryptu i edycji pracy. <i>Różalska</i> (podpis współautora)
dr Adrian Soboń	5	Współudział w opracowaniu metodyki i wykonaniu doświadczeń proteomicznych. <i>Soboń</i> (podpis współautora)
dr hab. Przemysław Bernat, prof. UŁ	15	Współudział w opracowaniu koncepcji pracy, projektowaniu metod, przeprowadzeniu badań. Ocena postępów, konsultacje metodyczne oraz uczestnictwo w przygotowaniu manuskryptu i edycji pracy, autor korespondujący. <i>Bernat</i> (podpis współautora)

4

Imię i Nazwisko	Szacunkowy udział [%]	Opis działań
mgr Julia Mironenka	75	Opracowanie koncepcji badań obejmujących zastosowanie metabolitów <i>Trichoderma</i> , jako związków potencjalnej ochrony pszenicy, w obecności czynników stresowych. Zaplanowanie i realizacja doświadczeń dotyczących wpływu <i>T. harzianum</i> i jego metabolitów zewnątrzkomórkowych na kiełkowanie pszenicy w obecności <i>F. culmorum</i> i 2,4D. Analiza zmian profilu białkowego pędów i korzeni. Ocena wpływu dodanych modeli grzybowych oraz herbicydu na stres oksydacyjny. Statystyczna analiza otrzymanych wyników. Przygotowanie podstawowej wersji manuskryptu, opracowanie graficzne wyników. Przygotowaniu odpowiedzi dla recenzentów. Julia Mironenka (podpis współautora)
dr hab. Sylwia Różalska, prof. UŁ	10	Współudział w opracowaniu koncepcji pracy, projektowanie metod, uczestnictwo w przygotowaniu manuskryptu, edycja pracy oraz udział w przygotowaniu odpowiedzi dla recenzentów. Rozalska (podpis współautora)
dr hab. Przemysław Bernat, prof. UŁ	15	Współudział w opracowaniu koncepcji pracy, projektowanie metod, ocena postępów, uczestnictwo w napisaniu manuskryptu a także udział w przygotowaniu odpowiedzi dla recenzentów, autor korespondujący. Bernat (podpis współautora)