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ROZPRAWA DOKTORSKA

stanowiąca cykl publikacji pod wspólnym tytułem

Zastosowanie substancji biologicznie czynnych celem ograniczenia toksyczności ołowiu w roślinie

The use of biologically active substances to reduce lead toxicity in the plant

Praca doktorska wykonana w Katedrze Bioinżynierii
Promotor
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Spis treści

1. Wstęp

Rośliny w całym swoim cyklu życiowym poddawane są różnym rodzajom stresów środowiskowych, do których należą m.in. zasolenie, niedobór wody, ekstremalne temperatury, promieniowanie UV, a także stężenie toksycznych pierwiastków, w tym metali ciężkich (Hayat i in. 2012). Metale ciężkie są jednymi z najważniejszych stresów środowiskowych (Hadia-e-Fatima 2018). Związki te występują naturalnie w skorupie ziemskiej w różnych stężeniach. Obecne w glebie utrzymują się przez dłuższy czas, powodując szkodliwe skutki zarówno dla środowiska, jak i organizmów żywych (Mansor i in. 2023, Hoque i in. 2021). Rośliny są w stanie pobierać metale ciężkie z gleby poprzez korzenie. Może to prowadzić do odkładania się ich w tkankach roślinnych, przez co roślina staje się toksyczna dla spożywających je zwierząt i ludzi (Iqbal i in. 2020).

Ołów (Pb) jest drugim najbardziej toksycznym metalem ciężkim w środowisku (Sharma i Dubey 2005). Jest szeroko stosowany w nowoczesnych gałęziach przemysłu do produkcji takich produktów, jak akumulatory kwasowo-ołowiowe, osłony radiacyjne, benzyna, farby, pestycydy, ceramika i chemikalia. Najwyższe poziomy tego pierwiastka w powietrzu występują najczęściej w pobliżu hut ołowiu. Inne obszary znajdują się w pobliżu spalarni śmieci, ścieków przemysłowych, producentów akumulatorów kwasowo-ołowiowych i miejsc stosowania nawozów fosforowych (Sharma i Dubey 2005, Shafiq i in. 2008, Kabir i in. 2010). Narażenie na ołów hamuje wzrost, biomasę i rozwój roślin oraz niekorzystnie wpływa na procesy fizjologiczne i biochemiczne (Shahid i in. 2014, Sędzik i in. 2015, Ghori i in. 2019, Awino i in. 2022, Hafeez i in. 2023). Następną konsekwencją jest stres oksydacyjny, który występuje w roślinach poprzez wytwarzanie reaktywnych form tlenu (RFT) (Shahid i in. 2014). Zwiększona produkcja RFT w roślinach podczas stresu może nasilać procesy oksydacyjne takie jak: peroksydacja lipidów błonowych, utlenianie białek, oraz hamowanie enzymów i uszkodzenia DNA i RNA (Asada 2006, (Shahzad i in. 2018). Aby pozbyć się RFT, rośliny posiadają wypracowany system obrony antyoksydacyjnej, który obejmuje zarówno składniki nieenzymatyczne, jak i enzymatyczne. Różne organelle w komórkach roślinnych, takie jak chloroplasty, mitochondria i peroksysomy, mają odrębne systemy wytwarzania i wychwytywania RFT. Procesy usuwania RFT w różnych przedziałach komórkowych są skoordynowane (Mansoor i in. 2023).

Zanieczyszczenie gleby ołowiem stanowi poważny problem dla środowiska, mający dalekosiężne konsekwencje dla rozwoju upraw zbóż. Uprawy te są niezbędne dla zrównoważonych systemów żywnościowych, ponieważ pochłaniają wodę i składniki odżywcze z gleby, potencjalnie pobierając przy tym ten toksyczny pierwiastek (Shahid 2017, Zakaria i in. 2021, Awino 2022, Javaid i in. 2022). Uprawy zbóż (pszenica, ryż, kukurydza i jęczmień) mają ogromne znaczenie dla

dostępności żywności, ponieważ stanowią podstawę światowych dostaw żywności, dostarczając znaczną część niezbędnych składników odżywczych niezbędnych do utrzymania człowieka (Vasilachi i in. 2023).

Według Organizacji Narodów Zjednoczonych ds. Wyżywienia i Rolnictwa (FAO) światowa produkcja zbóż w 2023 r. osiągnęła najwyższy w historii poziom 2836 mln ton. W uprawach rolnych w Polsce i Unii Europejskiej (UE) zboża stanowią również najważniejszą grupę roślin. Z danych GUS wynika, że w 2023 r. łączna powierzchnia przeznaczona pod uprawę zbóż wyniosła 7,2 mln ha (35,8 mln ton plonów), z czego sam jęczmień zajmował 0,6 mln ha.

Jęczmień *Hordeum vulgare* L. jest jedną z najstarszych roślin uprawnych. Uprawia się ją głównie z przeznaczeniem do spożycia przez ludzi, jako składnik pasz dla zwierząt oraz do produkcji napojów alkoholowych. Jest dobrym przedmiotem badań nad stresem abiotycznym ze względu na szybki wzrost, niskie wymagania klimatyczne, zdolność adaptacji do różnych środowisk i wyraźną reakcję na czynniki stresowe (Ullah i in.2016).

Aby poprawić tolerancję roślin lub złagodzić stres wywołany metalami ciężkimi pod względem fizjologicznym, biochemicznym i molekularnym w komórce, w licznych badaniach naukowych eksperymentowano z różnymi substancjami naturalnymi, zaliczanymi do substancji biologicznie czynnych, które wprowadzano egzogennie. Skuteczne łagodzenie stresu ołowiowego osiągnięto poprzez zastosowanie substancji takich jak brassinoidy (Soraes i in. 2020, Guedes i in. 2021, Khan i in. 2023, Emamverdian i in. 2024), auksyny, cytokininy (Piotrowska-Niczyporuk i in. 2020), kwas salicylowy (SA) (Arshad i in. 2017, Hasanuzzaman i in. 2019), kwas jasmonowy (JA) (Bali i in. 2018), różne chelaty organiczne (Khan i in. 2016, Saman i in. 2023), glutation (GSH) (Ahmad i in. 2023) i witaminy (Alamri i in. 2018, Sędzik-Wójcikowka i in. 2019, Sędzik-Wójcikowska i in. 2023).

Witaminy są związkami bioregulacyjnymi, które w stosunkowo niskich stężeniach wywierają głęboki wpływ na czynniki regulujące wzrost roślin, wpływające na wiele procesów fizjologicznych, takich jak synteza enzymów, działają jako koenzymy i wpływają na wzrost roślin. (Reda i in. 2005, Hassanein i in. 2009). Niacyna (witamina B) jest witaminą rozpuszczalną w wodzie, znana również jako nikotynamid lub kwas nikotynowy. Jest składnikiem koenzymów NAD (dinukleotyd nikotynamidoadeninowy) i NADP (fosforan dinukleotydu nikotynamidoadeninowego), niezbędnych w różnych procesach metabolicznych. Nikotynamid, pełniąc także funkcję obronną przy stresach środowiskowych (Khurshid i in. 2023).

W ramach pracy doktorskiej przeprowadzono próbę znalezienia najbardziej efektywnej substancji biologicznie czynnej, a także stężenia i metody aplikacji tej substancji, która zredukuje toksyczne działanie ołowiu na rośliny.

2. Cel badań

W ramach osiągnięcia naukowego, stanowiącego w rozumieniu ustawy Art. 15 Ust. 2 Ustawa z dnia 14 marca 2003 roku o stopniach naukowych i tytule naukowym oraz o stopniach i tytule w zakresie sztuki (Dz.U. 2016, poz. 882 ze zm.), przedstawiono jednotematyczny cykl publikacji.

Celem naukowym pracy doktorskiej było ograniczenie toksyczności stresu spowodowanego $Pb(NO_3)_2$ poprzez egzogenne zastosowanie substancji biologicznie czynnych.

Cel ten zrealizowano poprzez cele szczegółowe, którymi były:

- Wybór gatunku rośliny uznanej za wrażliwą na podstawie zmierzonych parametrów morfologicznych, fizjologicznych i biochemicznych 10-dniowych siewek różnych gatunków roślin uprawnych.
- Wybór substancji biologicznie czynnej, która w największym stopniu niweluje toksyczny wpływ ołowiu na parametry morfologiczne, biochemiczne i fizjologiczne w liściach 10 dniowego jęczmienia jarego odmiany *Eunova*, uznanego za gatunek wrażliwy na ołów.
- Egzogenne zastosowanie witaminy PP w celu zmniejszenia stresu wywołanego przez $Pb(NO_3)_2$ w roślinie wrażliwej, w doświadczeniu laboratoryjnym.
- Wybór najbardziej efektywnej metody stosowania substancji biologicznie czynnej zapobiegającej stresowi wywołanemu przez Pb w roślinie wrażliwej w dwuletnim doświadczeniu wazonowym.

3. Materiały i metody badań

Badania podzielono na 4 etapy i przeprowadzono je w latach 2014-2016 na Wydziale Kształtowania Środowiska i Rolnictwa w Zachodniopomorskim Uniwersytecie Technologicznym w Szczecinie. Pierwszy etap badań, przeprowadzono w warunkach laboratoryjnych w Katedrze Genetyki, Hodowli i Biotechnologii Roślin. Drugi etap przeprowadzono w laboratorium Katedry Bioinżynierii. Trzeci etap, doświadczenie *in vitro*, przeprowadzono w laboratorium Katedry Genetyki, Hodowli i Biotechnologii Roślin. Czwarty etap, 2-letnie doświadczenie wazonowe przeprowadzono w Hali Wegetacyjnej Wydziału Kształtowania Środowiska i Rolnictwa oraz w laboratorium Katedry Bioinżynierii.

3.1 Opis doświadczeń

3.1.1 I etap – doświadczenie laboratoryjne

Materiał do badań stanowiły niezaprawiane nasiona, 12 gatunków roślin powszechnie stosowanych w badaniach toksyczności (fitotoksyczności): dynia (*Cucurbita pepo*, odm. Danka Polka), rzodkiewka (*Raphanus dativus*, odm. Carmen), ogórek (*Cucumis sativus* L.), jęczmień (*Hordeum vulgare* odm. Eunova), żyto zwyczajne (*Secale graine* odm. Bojko), pszenica (*Triticum aestivum* L. odm. Bryza), łubin niebieski (*Lupinus angustifolius* L. odm. Karo), słonecznik (*Helianthus annuus*), pomidor (*Lycopersicon esculentum* odm. Faworyt), lucerna (*Medicago sativa* L.), rzeżucha zwyczajna (*Lepidium sativum*), soczewica (*Lens culinaris* odm. Medik).

Nasiona danych gatunków scharakteryzowano pod kątem fitotoksyczności wywołanej azotanem ołowiu $Pb(NO_3)_2$.

Przed założeniem doświadczenia nasiona roślin odkażono 70% (w/w) roztworem etanolu przez 30 sekund, a następnie przepłukano wodą dejonizowaną. Po wstępnej dezynfekcji nasiona moczono przez 15 minut w 10% (w/w) roztworze podchlorynu sodu (NaOCl), po czym trzykrotnie przepłukano sterylną wodą. Następnie nasiona umieszczono na szalkach Petriego wyłożonych (Ø10 cm) bibułą filtracyjną i zwilżonych wodą sterylną o objętości 30,0 cm^3 (kontrola) i 30 cm^3 1 mM $Pb(NO_3)_2$.

Płytki inkubowano w temperaturze 21°C w ciemności przez 72 godziny, po tym czasie obliczono liczbę nasion, które skiełkowały. Następnie rośliny testowe przeniesiono do fitotronu w Katedrze Genetyki, Hodowli i Biotechnologii Roślin Zachodniopomorskiego Uniwersytetu Technologicznego w Szczecinie i trzymano w warunkach o ściśle regulowanej temperaturze

(25°C), wilgotności (70–80%) i przy oświetleniu (około 40 $\mu E \cdot m^{-2} \cdot s^{-1}$). Podczas badania utrzymywano fotoperiod wynoszący 16:8 godzin.

Po 10 dniach wzrostu w roślinach oznaczono parametry morfologiczne (długość korzenia, długość pędu, świeża biomasa i wskaźnik tolerancji) i parametry fizjologiczne (prolina, chlorofil ogólny i karotenoidy).

Na podstawie wyników badań z etapu I wybrano rośliny uznane za tolerancyjne i wrażliwe na stres wywołany 1 mM $Pb(NO_3)_2$.

3.1.2 II etap – doświadczenie laboratoryjne

Materiał roślinny stanowiły nasiona jęczmienia jarego (*Hordeum vulgare* L.) odmiany Eunova, zakupione w specjalistycznym sklepie jako nasiona kwalifikowane w klasie (C/1).
W doświadczeniu tymoceniono wrażliwość jęczmienia jarego na działanie 1 mM $Pb(NO_3)_2$ oraz stopień łagodzenia toksyczności Pb poprzez egzogenne zastosowanie substancji biologicznie czynnych: kwasu askorbinowego (1 mM AsA), glutationu (100 μM GSH), nikotynamidu (50 μM PP), α-tokoferolu (1 mM α-Toc), kwasu salicylowego (1 mM SA) przeznaczonych na nasiona i sadzonki jęczmienia. Dezynfekcję nasion przeprowadzono według metody opisanej przez Krupę-Malkiewicz i in. (2018). W etapie II warunki doświadczenia były analogiczne jak w etapie I.

3.1.3 III etap – doświadczenie laboratoryjne in vitro

Materiał do badań stanowiły niezaprawione nasiona jęczmienia jarego (*Hordeum vulgare* odm. Eunova). Tolerancję na stres Pb oceniano poprzez pomiar cech morfologicznych (długość korzeni i pędów, świeżą masę roślin), parametrów biochemicznych i fizjologicznych (zawartość dialdehydu malonowego i proliny, aktywność katalazy, zawartość chlorofilu całkowitego i karotenoidów) 10-dniowych sadzonek pochodzących z zarodków hodowanych na pożywce MS (Murashige, Skoog 1962) zawierającej 0,5 - 2,0 mM soli Pb samej lub 25 - 100 μM nikotynamidu (wit PP). Zawartość ustalono na podstawie wcześniejszych badań (Sędzik i in. 2015). Pożywką kontrolną był MS. Doświadczenie przeprowadzono w 16 kombinacjach: 1) kontrola, 2) 25 μM Wit. PP, 3) 50 μM wit. PP, 4) 100 μM wit. PP, 5) 0,5 mM $Pb(NO_3)_2$, 6) 0,5 mM $Pb(NO_3)_2$ + 25 μM wit. PP, 7) 0,5 mM $Pb(NO_3)_2$ + 50 μM wit. PP, 8) 0,5 mM $Pb(NO_3)_2$ + 100 μM wit. PP, 9) 1 mM $Pb(NO_3)_2$, 10) 1 mM $Pb(NO_3)_2$ + 25 μM wit. PP, 11) 1 mM $Pb(NO_3)_2$ + 50 μM wit. PP, 12) 1 mM $Pb(NO_3)_2$ + 100 μM wit. PP, 13) 2 mM $Pb(NO_3)_2$, 14) 2 mM $Pb(NO_3)_2$ + 25 μM wit. PP, 15) 2 mM $Pb(NO_3)_2$ + 50 μM wit. PP, 16) 2 mM $Pb(NO_3)_2$ + 100 μM wit. PP.

Każdą kombinację doświadczenia reprezentowało 100 zarodków. Zarodki wypreparowano z nasion namoczonych w 0,5% kwasie siarkowym przez 20 minut, trzykrotnie przepłukanych sterylną wodą destylowaną. Następnie nasiona traktowano 7% roztworem podchlorynu sodu, płukano przez 15 minut w sterylnej wodzie destylowanej i moczono w wodzie przez 24 godziny. Zarodki wycięto igłą, trzymano je w 10% podchlorku sodu przez 10 minut, przepłukano sterylną wodą destylowaną i przeniesiono do odpowiedniej pożywki w probówkach (30 cm^3), w probówce (9 cm x 3,5cm) umieszczono po 4 zarodki. Probówki przykryto folią aluminiową i parafilmem i trzymano przez 10 dni w komorze wzrostowej w temperaturze 24°C, przy fotoperiodzie 16 godzin (40 µmol m^{-2} s^{-1}) i wilgotności względnej 55-60%.

3.1.4. IV etap – doświadczenie wazonowe

W tym etapie oceniono skutki łagodzenia stresu wywołanego przez 1mM $Pb(NO_3)_2$ poprzez dodanie amidu kwasu nikotynowego o stężeniu 100 µM w jęczmieniu jarym (*Hordeum vulgare* L.) odm. Eunova na podstawie pomiarów morfologicznych (długość kłosa, długość łodygi, długość korzenia, świeża), biochemicznych (CAT, POX proline, MDA) i fizjologicznych (chlorofil całkowity karotenoidy).

Dwuletnie doświadczenie wazonowe odbyło się wiosną 2015 i 2016 roku w Hali Wegetacyjnej WKŚiR i laboratorium Mikrobiologii i Biochemii Środowiska, (obecnie Bioinzynierii) Zachodniopomorskiego Uniwersytetu Technologicznego w Szczecinie. Gleba do badań pochodziła z warstwy orno-próchniczej (Ap, 0–30 cm) w Ostoi koło Szczecina. Glebę tę przesiano przez sito o średnicy oczek 2 mm i podzielono na osiem części. Do czterech części dodano 1 mM roztwór $Pb(NO_3)_2$ (207,0 mg Pb^{+2}), a do pozostałych części wprowadzono wodę, doprowadzając do 60% maksymalnej pojemności wodnej. Przygotowaną ziemię następnie umieszczono w doniczkach o masie 3,50 kg.

Materiał do badań stanowiły nasiona jęczmienia jarego odm. Eunova, nabyte jako kwalifikowany materiał siewny klasy (C/1) w wyspecjalizowanym sklepie. Przed wysiewem nasiona trzykrotnie moczono przez 20 minut w sterylnej wodzie destylowanej. Następnie zanurzano je w 7% roztworze podchlorynu sodu na 10 minut i płukano w sterylnej wodzie destylowanej przez 15 minut. Po wstępnym procesie sterylizacji część nasion zanurzono w roztworze 100 µM Wit PP w postaci nikotynamidu, a pozostałe nasiona moczono w wodzie przez 24 godziny. Do każdej doniczki na głębokość 3,0 cm wysiano taką samą liczbę nasion (10 nasion jęczmienia na doniczkę). Eksperyment składał się z 8 kombinacji przeprowadzonych w 3 powtórzeniach: 1) kontrola, 2) 100 µM Wit PP moczenie nasion, 3) 100 µM Wit PP oprysk, 4) 100 µM Wit PP podlewanie, 5) 1 mM

$Pb(NO_3)_2$, 6) 1 mM $Pb(NO_3)_2$ + 100 µM Wit PP moczenie nasion, 7) 1 mM $Pb(NO_3)_2$ + 100 µM Wit PP oprysk, 8) 1 mM $Pb(NO_3)_2$ + 100 µM Wit PP podlewanie.

Doniczki podlewano co 10 dni (100 cm^3 wody destylowanej) do momentu pojawienia się pierwszych liści. Następnie jęczmień jary odmiany Eunova podlewano co 10 dni (100 cm^3 wody destylowanej lub wody z dodatkiem 100 µM Wit PP). Dodatkowo niektóre rośliny opryskano roztworem zawierającym 100 µM Wit PP. Każdą roślinę w doniczce dokładnie spryskano roztworem, aż kapał z liści do doniczki, przy czym na każdą roślinę przypadało 10 cm^3 roztworu, co daje w sumie 100 cm^3 na doniczkę. Wszystkie roztwory Wit PP stosowane do oprysków i podlewania zawierały dodatek substancji Tween 20.

W okresie wegetacji obserwowano wzrost i rozwój roślin. Pomiary wykonano w czterech fazach rozwojowych jęczmienia jarego.

3.2 Metody

3.2.1. Metoda pomiaru cech morfologicznych

Pomiar cech morfologicznych badanych gatunków roślin przeprowadzono za pomocą linijki i podano w cm z dokładnością do 1 mm.

3.2.2 Metoda pomiaru wskaźnika kiełkowania

W trzeciej dobie doświadczenia obliczono indeks kiełkowania (GI%) badanych gatunków roślin. Za skiełkowane nasiona uznano te, które posiadały korzeń o długości min. 2 mm.
Obliczono go ze wzoru podanego przez Barbero i in. (2001):

$$IG\ \% = (GS\ Ls)/(Gc\ Lc) \times 100$$

gdzie:
Gs i Ls – kiełkowanie nasion i długość korzenia (mm) rośliny narażonej na stres Pb;
Gc i Lc – odpowiadające wartości dla kontroli.

3.2.3 Metoda pomiaru wskaźnika tolerancji (TI)

Wskaźnik tolerancji (TI) obliczono dzieląc długość korzenia rośliny narażonej na stres Pb przez długość zmierzoną podczas wzrostu w roztworze kontrolnym. Zastosowano następujące równanie:

TI (%) = 100 x (długość korzenia potraktowana Pb)/(długość korzenia w roztworze kontrolnym)

3.2.4 Metoda pomiaru świeżej masy roślin

Świeżą masę roślin w I i II etapie doświadczenia dokonano metodą wagową. Na wadze analitycznej zważono siewki z dokładnością do 0,001 g po 10 dniach wzrostu roślin.

3.2.5 Metoda oznaczania zawartości barwników asymilacyjnych w tkance roślinnej

Ekstrakcję barwników liści przeprowadzono 80% (w/w) acetonem. Chlorofil całkowity (chlorofil a+b) i zawartość karotenoidów oznaczono spektrofotometrycznie przy 663, 645 i 440 nm. Zawartość chlorofili mierzono według Arnona i in. (1956) w modyfikacji Lichtenthalera i Wellburna (1983), natomiast zawartość karotenoidów oznaczono metodą Hagera, Meyer-Berthenrath (1966). Stężenia chlorofilu a, b oraz karotenoidów przeliczono ze wzorów Arnona i wyrażono jako $\mu g \cdot g^{-1}$ ś.m. rośliny.

3.2.6 Metoda oznaczania zawartości wolnej proliny (Pro) w tkance roślinnej

Zawartość proliny mierzono metodą Batesa i in. (1973). Świeże sadzonki (0,5 g) zmielono w 1,5 ml wodnego roztworu kwasu sulfosalicylowego 3% (w/w) i oznaczono prolinę za pomocą kwaśnej ninhydryny. Próbki ekstrahowano toluenem. Absorbancję fazy toluenowej odczytano przy 520 nm. Stężenie proliny przeliczono z krzywej wzorcowej i wyrażono w $\mu mol\ Pro \cdot g^{-1}$ ś.m. rośliny.

3.2.7 Metoda oznaczania zawartości dialdehydu malonowego (MDA) w tkance roślinnej

Zawartość dialdehydu malonowego (MDA) jako produktu peroksydacji lipidów oznaczono kwasem tiobarbiturowym (TBA) metodą Sudhakara i in. (2001). Absorbancję mierzono przy 532

nm i 600 nm przy użyciu spektrofotometru. Zawartość MDA przeliczono z krzywej wzorcowej wyrażono w nmol MDA·g^{-1} ś.m. rośliny.

3.2.8 Metoda oznaczania aktywności katalazy

Aktywność katalazy (CAT) [EC 1.11.1.6] oznaczono za pomocą spektrofotometru zgodnie z metodą Lücka (1963). Test obejmował pomiar spadku absorpcji światła ultrafioletowego w ciągu 60 s w miarę rozkładu H_2O_2 za pomocą CAT przy długości fali λ = 240 nm. Aktywność enzymu wyrażono jako μM $H_2O_2 \cdot g^{-1}$ ś.m. rośliny · min^{-1}.

3.2.9 Metoda oznaczania aktywności peroksydazy

Aktywność peroksydazy (POX) [EC 1.11.1.7] mierzono metodą Chance'a i Maehly'ego (1955) przy użyciu spektrofotometru. Metoda polegała na kolorymetrycznym oznaczeniu powstawania purpurogaliny podczas utleniania pirogalolu (0,02 M) w obecności H_2O_2 (0,06 M) przy długości fali λ = 430 nm w czasie 4 min. Aktywność peroksydazy wyrażono w μM purpurogaliny · g^{-1} ś.m. rośliny · min^{-1}.

3.2.10 Analiza statystyczna

Otrzymane wyniki badań poddano analizie statystycznej za pomocą programu:
W I i III etapie badań istotność różnic określono za pomocą analizy wariancji i testu Tukeya, na poziomie istotności $\alpha = 0,05$. W kolejnym etapie analizy statystyczne przeprowadzono przy użyciu programu Statistica 13 (TIBCO Software Inc.). Uzyskane wyniki analizowano przy użyciu statystyki opisowej (średnia, odchylenie standardowe) i dwuczynnikowej ANOVA. Do porównania średnich wykorzystano test HSD Tukeya przy poziomie istotności $p < 0,05$. W IV etapie analizy statystyczne przeprowadzono przy użyciu programu Statistica 13 (TIBCO Software Inc.). Wyniki analizowano za pomocą statystyki opisowej, obejmującej średnią i odchylenie standardowe. Do porównania średnich wykorzystano test HSD Tukeya na poziomie istotności $p < 0,05$.

4. Omówienie wyników badań

W autoreferacie zostały zaprezentowane jedynie najistotniejsze wyniki, które miały wpływ na wyciągnięcie wniosków.

4.1 Etap I

Zastosowany w doświadczeniu $Pb(NO_3)_2$ wykazał toksyczny wpływ na kiełkowanie nasion, wzrost korzeni, wzrost siewek i suchą masę badanych gatunków roślin w odniesieniu do kontroli.

Rośliny testowe charakteryzowały się różnym indeksem kiełkowania. Wartości wskaźnika kiełkowania były najwyższe dla ogórka (40,40%), a najniższe dla słonecznika (9,20%). Zastosowanie $Pb(NO_3)_2$ miało istotny wpływ ($p \leq 0,05$) na spadek długości korzeni i największy stwierdzono w słoneczniku (90,14%), pomidorze (89,74%), lucernie (84,76%) i soczewicy (81,16%). Najmniej wrażliwa na Pb była dynia, żyto i pszenica.

Oceniając długość siewki badanych gatunków roślin, stwierdzono, że obecność $Pb(NO_3)_2$ powodowała zmniejszenie długości sadzonek w roślinach testowych w porównaniu z roślinami kontrolnymi. Największe zmniejszenie długości siewek zaobserwowano u lucerny (60,89%), słonecznika (45,43%) i pietruszki (44,19%). Efekt hamujący był bardziej wyraźny na długości korzenia niż na długości pędu. W badaniach zaobserwowano istotny spadek świeżej biomasy ($p \leq 0,05$) u roślin rosnących przy stężeniu Pb w zakresie od 8,4 do 66,7% w porównaniu do kontroli.

Po 10 dniach ekspozycji na Pb wartość TI u badanych gatunków roślin była niska, co oznacza dużą wrażliwość roślin testowych na ten pierwiastek. Wyższe wartości TI odnotowano w dyni, życie i pszenicy i wynosiły one odpowiednio 86,06, 68,76 i 60,03%.

Sól ołowiu wpłynęła na istotny statystycznie spadek ($p \leq 0,05$) barwników fotosyntetycznych – całkowitej zawartości chlorofilu i karotenoidów w liściach roślin w porównaniu z kontrolą.

4.1.1 Podsumowanie wyników I etapu badań

Zastosowane w doświadczeniu stężenie soli ołowiu hamowało kiełkowanie nasion, wzrost, zarówno korzeni, jak i długości pędów, świeżą masę roślin, zawartość barwników asymilacyjnych oraz podwyższało stężenie proliny w 10-dniowych siewkach badanych gatunków roślin uprawnych.

Spośród 12 przebadanych roślin wytypowano 3 gatunki, które wykazały tolerancję na ołów.

Do roślin tych zaliczono: dynię, żyto i pszenicę.

Najbardziej wrażliwe na obecność ołowiu okazały się: jęczmień jary, pomidor, lucerna i rzodkiewka. Do kolejnego etapu badań wybrano jęczmień jary.

4.2 Etap II

Analiza cech morfologicznych siewek jęczmienia pozwoliła stwierdzić, że $Pb(NO_3)_2$ średnio wpływał na zmniejszenie długości korzeni i koleoptylów oraz świeżej masy siewek w stosunku do kontroli odpowiednio o 81,8%, 36,6% i 41,2%. Zastosowanie egzogennych substancji biologicznie czynnych, takich jak: GSH i SA, zmniejszyło o 0,9% do 10,75% długość zarówno korzeni, jak i długość siewek w porównaniu do kontroli, chociaż nie wszystkie średnie różniły się istotnie.
Zastosowanie substancji biologicznie czynnych spowodowało zwiększenie świeżej masy siewek od 6,2% do 14,5% oraz wpłynęło na wzrost od 3,1% do 12,3% długość zarówno korzeni, jak i siewki w porównaniu do kontroli. Natomiast zastosowanie kombinacji substancji biologicznie czynnych z solą ołowiu spowodowało zmniejszenie toksycznego wpływu jonów ołowiu na kształtowanie się parametrów morfologicznych siewek jęczmienia. Największy wpływ łagodzący na hamowanie długości korzeni i siewek oraz świeżej masy sadzonek stwierdzono po zastosowaniu nikotynamidu. Stres ołowiu istotnie zwiększył zawartość MDA (o 176,7%) u siewek w porównaniu z kontrolą. Zastosowanie ołowiu w połączeniu z substancjami biologicznie czynnymi skutecznie obniżyło zawartość tego parametru w liściach jęczmienia. $Pb(NO_3)_2$ istotnie zwiększył również zawartość proliny, średnio o 153% w porównaniu do kontroli. Pod wpływem wszystkich zastosowanych substancji biologicznie czynnych zawartość proliny była wyższa w porównaniu do kontroli (H_2O) średnio o 7,9% (AsA), 18,0% (GSH), 3,0% (PP), 5,3% (α-Toc) i 27,7% dla SA, jedynie dla GSH i SA różnice te określono jako istotne.
Zastosowanie soli Pb istotnie obniżyło zawartość barwników fotosyntetycznych (chlorofilu całkowitego i karotenoidów) w badanych siewkach jęczmienia. Stres wywołany $Pb(NO_3)_2$ obniżył zawartość zarówno chlorofilu ogólnego, jak i karotenoidów o 33,5% i 31,3% w porównaniu do kontroli. Zaobserwowano jednak, że zastosowanie substancji biologicznie czynnych miało pozytywny wpływ na badane cechy w porównaniu z kontrolą. Zastosowanie wszystkich egzogennych substancji biologicznie czynnych w połączeniu z Pb istotnie zmniejszyło toksyczność jonów ołowiu w porównaniu z zawartością barwnika fotosyntetycznego w siewach traktowanych jedynie 1 mM $Pb(NO_3)_2$.

4.2.1 Podsumowanie wyników II etapu badań

Wyniki badań wykazały, że zastosowanie wszystkich egzogennych substancji biologicznie czynnych w połączeniu z $Pb(NO_3)_2$ istotnie zmniejszyło toksyczność jonów ołowiu. Amid kwasu nikotynowego (PP), α-tokoferol (wit. E) i glutation (GSH) w największym stopniu niwelują toksyczność ołowiu.

Z wybraną z doświadczenia II - „najskuteczniejszą" substancją biologicznie czynną, w tym przypadku amidem kwasu nikotynowego przeprowadzone zostało III doświadczenie w laboratorium *in vitro.*

4.3 Etap III

W niniejszym badaniu zastosowanie $Pb(NO_3)_2$ hamowało kiełkowanie nasion, wzrost korzeni i pędów oraz świeżą masę sadzonek jęczmienia w porównaniu z kontrolą.

Wartość wskaźnika kiełkowania nasion (%) jęczmienia jarego pod wpływem stresu $Pb(NO_3)_2$ wahała się od 16,77% do 31,40%. Zaobserwowano, że najniższa zdolność kiełkowania nasion jęczmienia występowała przy wyższym stężeniu soli ołowiu 2 mM $Pb(NO_3)_2$.

W przypadku siewek jęczmienia rosnących w pożywce MS z roztworem $Pb(NO_3)_2$ o stężeniu 1,0 i 2,0 mM największą skuteczność w łagodzeniu skutków stresu wykazała pożywka z dodatkiem witaminy PP w najwyższym stężeniu – 100 µM. Wyniki wykazały zmniejszenie długości i masy siewek jęczmienia dla obu stężeń $Pb(NO_3)_2$ w pożywce MS. Przy 2 mM $Pb(NO_3)_2$ w pożywce MS u 10-dniowych sadzonek zaobserwowano aż do 65% redukcję długości korzeni i 78% redukcję długości pędów w porównaniu z kontrolą. Podobnie, w przypadku sadzonek zaobserwowano aż do 59% spadek świeżej masy roślin w porównaniu z kontrolą przy 10-dniowym wzroście.

Stres ołowiu znacząco zwiększył zawartość MDA (od 27,47 do 32,09 nmol g^{-1} fm), a przy 2,0 mM $Pb(NO_3)_2$ efekt był wyraźniejszy. Zastosowanie nikotynamidu (niezależnie od stężenia) osłabiło wpływ stresu ołowiu, obniżając poziom MDA w porównaniu z kontrolą (22,90 nmol g^{-1} ś.m. rośliny).

Stwierdzono istotny wzrost zawartości proliny (1,41 µmol g^{-1} ś.m. rośliny) przy zastosowaniu 2,0 mM $Pb(NO_3)_2$ w porównaniu do kontroli (1,04 µmol g^{-1} ś.m. rośliny).

Warunki stresu metalami ciężkimi istotnie obniżyły poziom proliny w 10-dniowych siewach jęczmienia (0,55 – 0,96 µmol g^{-1} ś.m. rośliny)

W doświadczeniu zaobserwowano wzrost aktywności katalazy (CAT) podczas 10-dniowego wzrostu sadzonek jęczmienia pod wpływem stresu metalami ciężkimi. Wraz ze wzrostem poziomu $Pb(NO_3)_2$ w pożywce MS, aktywność CAT osiągnęła najwyższy poziom (63,87 µM H_2O_2 g^{-1} fm) w porównaniu do kontroli (48,71 µM H_2O_2 g^{-1} fm). Aktywność tego enzymu znacząco zmniejszyła się (41,42 - 59,98 µM H_2O_2 g^{-1} fm) w przypadku zastosowania nikotynamidu.

W przeprowadzonym etapie badań zwiększenie zawartości $Pb(NO_3)_2$ w pożywce MS spowodowało zmniejszenie całkowitego stężenia chlorofilu i karotenoidów w 10-dniowych siewkach jęczmienia. Jednakże dodanie nikotynamidu do pożywki MS uzupełnionej 1,0 i 2,0 mM $Pb(NO_3)_2$ miał znaczący wpływ na chlorofil i karotenoidy.

4.3.1 Podsumowanie wyników III etapu badań

Zastosowanie nikotynamidu jako przeciwutleniacza wpłynęło na wzrost, rozwój i parametry biochemiczne *Hordeum vulgare* odm. Eunova w kulturze *in vitro*. Najlepszy efekt łagodzący szkodliwe działanie ołowiu wykazano dla stężeń 50 i 100 μM nikotynamidu.

4.4 Etap IV

Analiza statystyczna parametrów morfologicznych jęczmienia przeprowadzona po fazie kwitnienia wykazała, że zarówno w pierwszym, jak i drugim sezonie, a także w zbiorczych danych z obu sezonów, nie stwierdzono istotnych różnic w mierzonych parametrach pomiędzy badanymi kombinacjami. Jedynie zaobserwowano istotne różnice w długości korzeni i pędów pomiędzy badanymi kombinacjami. Dodatek ołowiu spowodował zmniejszenie długości korzeni o 13,9% w pierwszym sezonie i o 19,9% w drugim sezonie, przy czym długość pędów zmniejszyła się odpowiednio o 24,8% i 16,2% w porównaniu z kontrolą. Ponadto zastosowanie egzogennej witaminy PP miało istotny i pozytywny wpływ na długość korzeni i pędów, łagodząc toksyczność soli ołowiu. Najbardziej efektywnym zastosowaniem witaminy PP był oprysk dolistny, który wykazał jej korzystny wpływ na wzrost roślin i redukcję stresu ołowiowego. Zaobserwowano, że aktywność enzymów biorących udział w obronie antyoksydacyjnej istotnie wzrosła ($p > 0,05$) w obecności soli ołowiu. Aktywność CAT wzrosła z 45% do 106% w porównaniu do kontroli, natomiast aktywność POX wykazywała podobny poziom wzrostu (z 39% do 46%) w fazach rozwojowych badanego jęczmienia (w obu latach). Jednakże zastosowanie witaminy PP do roślin na glebie skażonej ołowiem doprowadziło do obniżenia poziomu enzymów w porównaniu z roślinami narażonymi na działanie samego ołowiu. Najbardziej znaczący spadek aktywności enzymów zaobserwowano podczas oprysków dolistnych i nawadniania witaminą PP zarówno w pierwszym, jak i drugim roku badań.

Stres ołowiu w istotny sposób ($p > 0,05$) przyczynił się do wzrostu zawartości MDA w porównaniu z kontrolą (wzrost z 61% do 79,4%) w badanych stadiach rozwojowych jęczmienia odm. Eunova (w obu latach). Egzogenna aplikacja witaminy PP, szczególnie w formie podlewania i oprysku, istotnie ($p > 0,05$) obniżyła zawartość MDA w porównaniu do roślin rosnących z samym ołowiem.

Wprowadzona sól ołowiu przyczyniła się do wzrostu zawartości proliny w obu latach doświadczenia. Jednakże istotne różnice zaobserwowano jedynie w fazie kłoszenia i kwitnienia jęczmienia, przy czym nie stwierdzono znaczących różnic w zawartości Pro we wcześniejszych fazach rozwoju rośliny. Zastosowanie witaminy PP spowodowało zmniejszenie zawartości Pro w jęczmieniu odmiany Eunova. Najistotniejszy efekt zaobserwowano podczas opryskiwania i nawadniania.

Zastosowane w doświadczeniu sole ołowiu istotnie ($p > 0,05$) zmniejszyły zawartość barwników asymilacyjnych, w tym chlorofilu ogólnego i karotenoidów. W miarę wzrostu roślin następował stopniowy spadek zawartości chlorofilu ogólnego (od 20,3% do 35,3% niższej niż w kontroli), natomiast zawartości karotenoidów od 22,4% do 28,7% (w obu latach). Witamina PP, stosowana we wszystkich formach, zwiększała zawartość zarówno chlorofilu całkowitego, jak i karotenoidów w porównaniu do roślin rosnących z samym ołowiem.

4.4.1 Podsumowanie wyników IV etapu badań

Zastosowanie w doświadczeniu 1 mM $Pb(NO_3)_2$ spowodowało zmniejszenie długości korzeni i pędów oraz wzrost aktywności katalazy i peroksydazy, a także zawartości dialdehydu malonowego, proliny i barwników asymilacyjnych w badanych fazach rozwojowych jęczmienia jarego odmiany Eunova. Witamina PP miała istotny i korzystny wpływ na badane parametry morfologiczne, biochemiczne i fizjologiczne, zmniejszając tym samym toksyczność soli ołowiu.

5.Wnioski

Na podstawie przeprowadzonych badań ustalono następujące wnioski:

- 1 mM $Pb(NO_3)_2$ hamuje kiełkowanie nasion, wzrost korzeni, długości pędów, świeżą masę roślin, zawartość barwników asymilacyjnych oraz podwyższa stężenie proliny w 10-dniowych siewkach badanych gatunków roślin uprawnych. Wśród badanych roślin trzy gatunki, (dynia, żyto i pszenica) charakteryzują się zwiększoną tolerancją na 1 mM $Pb(NO_3)_2$, natomiast jęczmień jary, pomidor, lucerna i rzodkiewka są najbardziej wrażliwe.

- Spośród badanych substancji biologicznie czynnych najlepsze efekty w łagodzeniu negatywnych skutków stresu wywołanego 1 mM $Pb(NO_3)_2$ u jęczmienia jarego odm. Eunova wykazały: witamina PP, α-Tocoferol i glutation. Natomiast najniższe określono dla kwasu salicylowego.

- Dawka 50 i 100 μM witaminy PP zastosowana w doświadczeniu w kulturze *in vitro* przyniosła najlepsze rezultaty w zmniejszaniu szkodliwego wpływu ołowiu na jęczmień jary odmiany Eunova. Obserwowane złagodzenie toksyczności 1 mM $Pb(NO_3)_2$ po zastosowaniu witaminy PP u badanej rośliny w warunkach *in vitro* jest podobne jak w doświadczeniu wazonowym.

- Efekt zastosowanej dawki witaminy PP zależy od formy podania. Najlepszy efekt w ograniczaniu stresu ołowiowego daje oprysk dolistny i podlewanie witaminą PP.

- Badania dają perspektywę wykorzystania witaminy PP do poprawy odporności roślin na stres ołowiu.

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7. Streszczenie

Rośliny podczas swojego życia cały czas narażone są na działanie czynników abiotycznych jak i biotycznych. Zakłócają one funkcjonowanie roślin, negatywnie wpływając na procesy fizjologiczne i biochemiczne, co przejawia się w ograniczonym wzroście i plonowaniu. Spośród czynników abiotycznych wpływających na wzrost i rozwój roślin duże znaczenie odgrywają metale ciężkie. Niebezpieczeństwo zanieczyszczenia środowiska metalami ciężkimi wzrosło wskutek szybkiego rozwoju przemysłu na całym świecie. Ołów jest silnie toksycznym i trwale zanieczyszczającym środowisko metalem ciężkim. Jego związki wpływają negatywnie na przebieg szeregu procesów metabolicznych w roślinach.

Badania nad wykorzystaniem substancji biologicznie czynnych w rolnictwie mogą przynieść pozytywne wyniki w zakresie tolerancji roślin na stresy środowiskowe i zwiększenia ich odporności na szkodliwe działanie metali ciężkich. GSH chroni rośliny przed uszkodzeniami oksydacyjnymi powodowanymi przez czynniki stresowe. Jest obecny we wszystkich komórkach roślinnych (Foyer i Noctor 2005). Kwas salicylowy (SA) należy do grupy związków fenolowych. Jest fitohormonem pełniącym w roślinach funkcję sygnalizacyjną (Miura i Tada, 2014). Witaminy w dość niskich stężeniach wywierają głęboki wpływ na rośliny. Egzogenne podawanie witamin do roślin reguluje niekorzystny wpływ stresów abiotycznych na wzrost roślin, a także procesy fizjologiczne i biochemiczne. Nikotynamid (witamina PP) to rozpuszczalna w wodzie witamina z grupy B. Nikotynamid jest składnikiem koenzymów dinukleotydu pirydynowego NADH i NADPH, które biorą udział w wielu enzymatycznych reakcjach utleniania-redukcji w żywych komórkach (Abdelhamid i in. 2013, Azooz i in. 2013).

Celem naukowym pracy doktorskiej było ograniczenie toksyczności stresu spowodowanego $Pb(NO_3)_2$ poprzez egzogenne zastosowanie substancji biologicznie czynnych.

Cele zrealizowano poprzez 4 etapy badań: 3 doświadczenia laboratoryjne, w tym 1 *in vitro* i 1 dwuletnie wazonowe.

W etapie I określono skutki działania 1 mM $Pb(NO_3)_2$ na kształtowanie się parametrów morfologicznych i fizjologicznych w liściach 10-dniowych siewek w różnych gatunkach roślin uprawnych. Na podstawie wyników badań z 1 etapu wybrano do kolejnych etapów badań jęczmień jary (*Eunova*) jako roślinę wrażliwą.

W Etapie II oceniono skutki łagodzenia stresu wywołanego przez 1 mM $Pb(NO_3)_2$ poprzez zastosowanie substancji biologicznie czynnych na podstawie parametrów morfologicznych, biochemicznych i fizjologicznych w liściach 10 dniowego jęczmienia jarego odmiany *Eunova,* oraz

wybór substancji biologicznie czynnej, która w największym stopniu niweluje toksyczny wpływ ołowiu. Spośród badanych substancji biologicznie czynnych.

Etap III polegał na wyborze dawki Pb 0,5 - 2 mM $Pb(NO_3)_2$ oraz dawki substancji biologicznie czynnej (25, 50, 100 μM PP), przy których widać najwyraźniejszą reakcję jęczmienia jarego na podstawie parametrów morfologicznych, biochemicznych i fizjologicznych. Wykazano, że narażenie siewki jęczmienia na ołów wywołuje liczne zaburzenia metaboliczne. Stwierdzono właściwości przeciwutleniające i rolę nikotynamidu w stresie ołowiu, ponieważ związek ten może hamować szkodliwe działanie ołowiu. Zaobserwowano, że najlepszy efekt daje nikotynamid w stężeniach 50 lub 100μM.

Etap IV polegał na przeprowadzeniu oceny egzogennego zastosowania 100 μM amidu kwasu nikotynowego (witamina PP) wprowadzonego w formie: oprysku, moczenia nasion i podlewania przy stresie wywołanym 1 mM Pb w różnych fazach rozwojowych rośliny. Uzyskane wyniki wykazały, że egzogenna witamina PP miała znaczący i korzystny wpływ na badane parametry morfologiczne, biochemiczne i fizjologiczne, zmniejszając toksyczność soli ołowiu. Najlepszy efekt w ograniczaniu stresu ołowiowego osiąga się poprzez opryskiwanie dolistne i podlewanie witaminą PP. Badania dają perspektywę wykorzystania witaminy PP do poprawy odporności roślin na stres ołowiu.

Słowa kluczowe: jęczmień; ołów stres; substancje biologicznie czynne; nikotynamid; parametry morfologiczne, biochemiczne.

8. Abstract

The danger of environmental pollution with heavy metals has increased due to the rapid development of industry around the world. During their life, plants are constantly exposed to abiotic and biotic factors. They disrupt the functioning of plants, negatively affecting physiological and biochemical processes, which manifests itself in limited growth and yield. Among the abiotic factors influencing the growth and development of plants, heavy metals play an important role. Lead is a highly toxic and permanently polluting metal. Its compounds negatively affect the course of a number of metabolic processes in plants.

Research on the use of biologically active substances in agriculture may bring positive results in terms of plants' tolerance to environmental stresses and increasing their resistance to the harmful effects of heavy metals. Vitamins in fairly low concentrations have a profound effect on plants. Exogenous administration of vitamins to plants regulates the adverse effects of abiotic stresses on plant growth, as well as physiological and biochemical processes. Nicotinamide (vitamin PP) is a water-soluble vitamin from the B group. Nicotinamide is a constituent of the pyridine dinucleotide coenzymes NADH and NADPH, which are involved in many enzymatic oxidation-reduction reactions in living cells (Abdelhamidet al. 2013, Azoozet al. 2013).

The scientific aim of the doctoral thesis was to reduce the toxicity of stress caused by Pb(NO3)2 through the exogenous application of biologically active substances.

The goals were achieved through 4 stages of research: 3 laboratory experiments and one pot experiment.

In stage I, the effects of 1 mM lead nitrate on the development of morphological and physiological parameters in the leaves of 10-day-old seedlings in various crop plant species were determined. Based on the test results from stage 1, spring barley (Eunova) was selected for subsequent stages of research.

In Stage II, the effects of alleviating stress caused by 1 mM lead nitrate were assessed through the use of biologically active substances based on morphological, biochemical and physiological parameters in the leaves of 10-day-old spring barley variety Eunova, and the selection of the biologically active substance that eliminates the toxic effect of lead to the greatest extent.

Stage III consisted in selecting the Pb dose of 0.5 - 2 mM $Pb(NO_3)_2$ and the dose of the biologically active substance (25, 50, 100 μM PP), which shows the clearest reaction of spring barley based on morphological, biochemical and physiological parameters. It has been shown that exposure of barley seedlings to lead causes numerous metabolic disorders. Antioxidant properties and a role for nicotinamide in lead stress have been found, as this compound can inhibit the harmful

effects of lead. It was observed that nicotinamide gives the best effect at concentrations of 50 or 100 μM.

Stage IV consisted in assessing the exogenous use of 100 μM nicotinic acid amide (vitamin PP) introduced in the form of: spraying, soaking seeds and watering under stress caused by 1 mM Pb in various stages of plant development. The obtained results showed that exogenous vitamin PP had a significant and beneficial effect on the tested morphological, biochemical and physiological parameters, reducing the toxicity of lead salts. The best effect in reducing lead stress is achieved by foliar spraying and watering with vitamin PP. The research provides the prospect of using vitamin PP to improve plant resistance to lead stress.

Keywords: barley; lead stress; biologically active substances; nicotinamide; morphological and biochemical parameters.

9. Kopie publikacji naukowych wchodzących w zakres rozprawy doktorskiej

Wykaz publikacji stanowiący wskazanie osiągnięcia zgodnie z Art. 15 Ust. 2 Ustawy z dnia 14 marca 2003 roku o stopniach naukowych i tytule naukowym oraz stopniach i tytule w zakresie sztuki Dz.U. 2016, poz 882 pod tytułem:

Zastosowanie substancji biologicznie czynnych celem ograniczenia toksyczności ołowiu w roślinie

Tab.1. Zestawienie punktowe dorobku naukowego

Lp.	Publikacja	IF*	Punktacja MNiSW **
1.	**Sędzik M.**, Smolik B., Krupa-Małkiewicz M. 2015. Effect of lead on germination and some morphological and physiological parameters of 10-day-old seedlings of various plant species. Ochrona Środowiska I Zasobów Naturalnych, 26(3): 22-27.	-	**40**
2.	**Sędzik-Wójcikowska M.**, Smolik B., Krupa-Małkiewicz M 2019. Effect of nicotinamide in alleviating stress caused by lead in spring barley seedling. Journal of Elementology, 24(1): 281-291.	**0,7**	**70**
3.	**Sędzik-Wójcikowska M.**, Krupa-Małkiewicz M., Smolik B. 2023. The Effect of Use of the Biologically Active Substances in Alleviating the Stress Caused by Lead in Barley Seedling on the Basis of Biochemical and Physiological Parameters. Journal of Ecological Engineering, 24(8).	**1,3**	**70**
4.	Smolik B.,**Sędzik-Wójcikowska M.**, 2024 Effect of Application Methods of Nicotinamide on the Alleviation of Lead-Induced Stress in Spring Barley. Agronomy, 14(6): 1314.	**3,7**	**100**
Razem		**5,7**	**280**

* Impact Factor (IF) wg bazy Journal Citation Reports (JCR) z roku wydania

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Effect of lead on germination and some morphological and physiological parameters of 10-day-old seedlings of various plant species

Wpływ ołowiu na kiełkowanie i niektóre parametry morfologiczne i fizjologiczne w 10-dniowych siewkach różnych gatunków roślin

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Keywords: heavy metal, lead, growth, proline, photosynthetic pigments
Słowa kluczowe: metale ciężkie, ołów, wzrost, prolina, barwniki fotosyntetyczne

Abstract
Among the heavy metals, lead (Pb) is one of the most common environmental pollutants. This study examines the effect of 1 mM lead nitrate $Pb(NO_3)_2$ on the germination index, morphological parameters (root length, shoot length, fresh biomass and tolerance index) and physiological parameters (proline, total chlorophyll and carotenoids) in the leaves of 10-day-old seedlings of various species of crop plants under laboratory conditions. All results, when compared to control, showed Pb adversely affecting the morphological and physiological parameters of the test plants. Among the 12 studied plants, three species (pumpkin, rye and wheat) presented high tolerance to Pb compared to the other test plants. The most sensitive to Pb exposure were radish, barley, tomato and alfalfa.

Streszczenie
Wśród metali ciężkich, ołów (Pb) jest jednym z najczęstszych zanieczyszczeń środowiska. Celem pracy było określenie wpływu 1mM $Pb(NO_3)_2$ – na indeks kiełkowania, a także na kształtowanie się parametrów morfologicznych (długość korzenia, długość pędu, świeżą masę, indeks tolerancji) oraz parametrów fizjologicznych (stężenie proliny, chlorofilu całkowitego i karotenoidów) w liściach 10 dniowych sadzonek różnych gatunków roślin uprawnych w warunkach laboratoryjnych. Ołów niekorzystnie wpływał na parametry morfologiczne i fizjologiczne testowych roślin. Spośród 12 badanych roślin trzy gatunki (dynia, żyto i pszenica) charakteryzowały się wysoką tolerancją na Pb w porównaniu do pozostałych badanych roślin. Najbardziej wrażliwe na działanie ołowiu były rzodkiewka, jęczmień, pomidor i lucerna.

1. INTRODUCTION

Pollution by heavy metals is one of the most important problems worldwide.

Among the heavy metals, lead (Pb) is a highly toxic and persistent environmental pollutant derived from various sources [Sharma and Dubey 2005]. Pb has been extensively used in modern industries to manufacture products such as lead-acid batteries, radiation shields, gasoline, paint, pesticides, ceramics and chemicals. The highest levels of Pb in air are generally found near Pb smelters. Other areas are near industrial effluents, waste incinerators, lead–acid battery manufacturers and while applying phosphate fertilisers [Sharma and Dubey 2005, Shafiq *et al*. 2008, Kabir *et al*. 2010].

Pb is a toxic environmental contaminant that induces many morphological, physiological and biochemical changes in plants. Pb toxicity leads to decreases in the percentage of seed germination [Shafiq *et al*. 2008] and in the growth and yield of plants [Shafiq *et al*. 2008, Kumar and Jayaraman 2014], disruption of mineral nutrition [Lamhamdi *et al*. 2013, Nareshkumar *et al*. 2014], inhibition of photosynthesis [Tian *et al*. 2014], inhibition of enzyme activity [Malar *et al*. 2014], water imbalance and alterations in membrane permeability [Sharma and Dubey 2005, Israr and Sahi 2008]. According to Wang [1987], germination and root growth are stages in plant development that are especially sensitive to contamination. Therefore, these stages may be observed for a fast biological assessment of environmental contamination as well as to make a preliminary assessment, selection and characterisation of a contamination-resistant species of crop plants [Wang and Williams 1988]. Cultivation of varieties that are tolerant to various environmental stressors is the easiest and cheapest way to counteract crop losses caused by them [Ashraf and Harris 2005]. Selecting cultivars tolerant to abiotic stresses contribute to improving crop yields.

The aim of this study is to determine the effect of 1 mM lead nitrate - $Pb(NO_3)_2$ on the morphological and physiological parameters of the leaves of 10-day-old seedlings of various species of crop plants, and assessment of sensitivity to the presence of Pb ions in the environment, which is the basis for the selection of certain plant species tolerant to unfavourable environmental conditions.

2. MATERIALS AND METHODS

The experiment was conducted in January 2014 in the laboratory of the Department of Plant Physiology and Biochemistry and also Department of Plant Genetics, Breeding and Biotechnology at the West Pomeranian University of Technology in Szczecin, Poland. The study material included undressed seeds collected from 12 plant species commonly used in testing the toxicity (phytotoxicity): pumpkin (*Cucurbita pepo,* var. 'Danka Polka'), radish (*Raphanus dativus,* var. 'Carmen'), cucumber (*Cucumis sativus* L.), barley (*Hordeum vulgare* var. 'Eunova'), rye ordinary (*Secale cereale* var. 'Bojko'), wheat (*Triticum aestivum* L. var. 'Bryza'), blue lupine (*Lupinus angustifolius* L. var. 'Karo'), sunflower *(Helianthus annuus),* tomato (*Lycopersicon esculentum* var. 'Faworyt'), alfalfa (*Medicago sativa* L.), cuckooflower (*Lepidium sativum*), lentil (*Lens culinaris* Medik.).

Seeds of each species were characterised towards Pb-induced phytotoxicity — lead nitrate [$Pb(NO_3)_2$]. Seeds were surface-sterilised with 70% (v/v) ethanol solution for 30 seconds and then thoroughly rinsed with sterile water. After the preliminary disinfection, the seeds were soaked for 15 minutes in 10% (v/v) solution of sodium hypochlorite (NaOCl), after rinsing three times in sterile water. Next, seeds were placed in petri dishes lined (Ø10 cm) with filter paper and moistened with 30.0 cm^3 sterile water (control) and of Pb solution. The experiment was established in three replications of 10 seeds in duplicate. The experiments were repeated three times.

The plates were incubated at 21°C in the dark for 72 hours, then the germinated seeds were counted and the root length measured. With both values, calculated Index of Germination (IG%) from the formula given by Barbero *et al*. [2001]:

$$IG\ \% = (GS\ Ls)/(Gc\ Lc) \times 100$$

where:

Gs and Ls - the seed germination and root length (mm) of the plant exposed to stress Pb;

Gc and Lc - the corresponding values for controls.

Then, the test plants were transferred to phytotron at the Department of Plant Genetics, Breeding and Biotechnology, West Pomeranian University of Technology in Szczecin, Poland and kept in highly regulated thermal (25°C), humidity (70–80%) and light (around 40 $\mu E \cdot m^{-2} \cdot s^{-1}$) conditions. During the study a 16:8 hour photoperiod was maintained.

After 10 days of growth, the effect of 1 mM $Pb(NO_3)_2$ on the morphological parameters (root length, shoot length, fresh biomass and tolerance index) and physiological parameters (proline, total chlorophyll and carotenoids) of 10-day-old seedlings of various species of crop plants were determined.

The *tolerance index (TI)* The tolerance index (TI) was calculated by dividing the root length of the plant exposed to stress Pb by that measured during growth in the control solution. The following equation was used:

TI (%) = 100 x (root length under Pb treatment)/(root length in the control solution).

Determination of proline Proline content was measured according to the method of Bates *et al*. [1973]. Fresh seedlings (0.5 g) were ground in 1.5 ml of aqueous sulfosalicylic acid 3% (w/v), and proline was estimated by ninhydrin reagent. The samples extracted with toluene and the absorbance of the toluene phase was read at 520 nm. The concentration of proline was calculated from a standard curve and expressed as $\mu mol \cdot g^{-1}$ fresh weight.

Determination of pigments The extraction of leaf pigments was performed with 80% (v/v) acetone. Chlorophyll a, b and carotenoids content was determined spectrophotometrically at 663, 645 and 440 nm. The concentration of total chlorophyll were calculated according to the method of Arnon *et al*. [1956] in modification to Lichtenthaler and Wellburn [1983], whereas the concentration of carotenoids was calculated according to the method of Hager and Meyer-Berthenratha [1966]. The pigment concentrations were expressed as $\mu g \cdot g^{-1}$ fresh weight.

Statistical analysis The significance of differences was determined by means of variance analysis and Tukey's test, at the level of significance of $\alpha = 0.05$.

3. RESULTS AND DISSCUSION

Growth inhibition is a common response of plants to heavy metal stress and is also one of the most important agricultural indices of heavy metal tolerance [Jiang and Liu 2010]. Pb toxicity has become important because of the steadily increasing levels of this metal in the environment. The effect of Pb on seedling growth seems to be different with regard to plant species, cultivars, organs and metabolic processes [Sharma and Dubey 2005].

Pb treatment showed toxic effect on seed germination, root growth, seedling growth and dry biomass of plant species tested with respect to control (Figure 1 and table 1). The test plants had various index of germination (Fig. 1). The values of index of germination were the highest for cucumber (40.40%), and the lowest for sunflower (9.20%).

Seed germination inhibitions by heavy metals have been reported by some researchers [Mahmood *et al*. 2005, Jamal *et al*. 2006]. These decreases in germination may be due to the interference of Pb with metabolic processes, which loss of viability decrease of energy generation for on embryo. Energy generation is very important for seed germination and its blockage affects protein, nucleic acids production, as well as mitosis [John and van-Laerhoven 1976].

The application of lead nitrate had a significant effect on decrease ($p \leq 0.05$) of root length in the studied plant species compared to the control plants. The highest decrease of root length was found in sunflower (90.14%), tomato (89.74%), alfalfa (84.76%) and lentil (81.16%) (Table. 1). The least sensitive to Pb was pumpkin, rye and wheat. In case of pumpkin and rye, no statistically significant difference was observed among root lengths from comparable culture conditions.

Evaluating seedling length in the studied plant species, it was found that the presence of Pb resulted in decrease in seedling length in the test plants compared to the control plants. The greatest reduction in seedling length was observed in alfalfa (60.89%), sunflower (45.43%) and parsley (44.19%) (Table 1). On the other

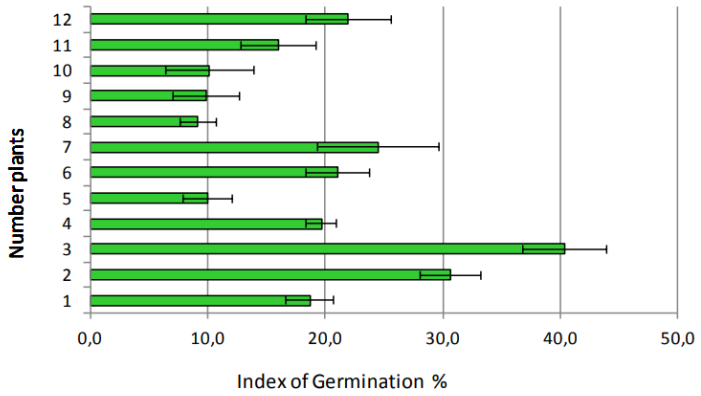

Figure 1. The value of IG% of seedlings of various species of plants growing under stress conditions induced with 1 mM Pb salt. The error bars indicate mean ± SD (n = 3); Nambers 1–12 are: 1 - pumpkin, 2 - radish, 3 - cucumber, 4 - barley, 5 - rye ordinary, 6 - wheat, 7 - blue lupine, 8 - sunflower, 9 - tomato, 10 - alfalfa, 11 - cuckooflower and 12 - lentil; IG%: index of germination.

Table. 1. Summary of morphological features and the fresh weight of seedlings of different species of plants growing under conditions of stress induced with 1 mM Pb salt

Plant species	Root length [cm]			Shoot length [cm]			Fresh weight [g]			Tolerance index [%])
	Control	1 mM soli Pb	$LSD_{0.05}$	Control	1 mM soli Pb	$LSD_{0.05}$	Control	1 mM soli Pb	$LSD_{0.05}$	
Pumpkin (*Cucurbita pepo*, var. 'Danka Polka')	6.53 ± 1.32 (100)	5.67 ± 1.25 (86.83)	n.d.	4.96 ± 0.65 (100)	4.7 ± 1.47 (94.76)	n.d.	1.4 ± 0.35 (100)	1.2 ± 0.06 (85.71)	n.d.	86.09
Radish (*Raphanus dativus*, var. 'Carmen')	3.22 ± 1.74 (100)	0.99 ± 0.34 (30.75)	1.39*	2.51 ± 1.12 (100)	1.75 ± 0.614 (69.72)	0.62*	0.07 ± 0.03 (100)	0,08 ± 0,04 (114,29)	n.d.	30.75
Cucumber (*Cucumis sativus* L.)	8.51 ± 2.53 (100)	3.44 ± 2,41 (40.42)	2.91*	2,17 ± 0,51 (100)	1,62 ± 0,50 (74,65)	0.41*	0.12 ± 0.07 (100)	0,11 ± 0,06 (91,67)	0.05*	40.42
Barley (*Hordeum vulgare* var. 'Eunova')	8.23 ± 1.61 (100)	1.83 ± 0.92 (22.24)	1.29*	9.98 ± 1.6 (100)	6.84 ± 0.64 (68.54)	0.84*	0.24 ± 0.06 (100)	0.16 ± 0.03 (66.67)	0.03*	22.24
Rye ordinary (*Secale cereale* var. 'Bojko')	8.58 ± 2.32 (100)	5.90 ± 2.32 (68.76)	n.d.	7.46 ± 2.31 (100)	4.84 ± 1.35 (64.88)	1.95*	0.17 ± 0.07 (100)	0.13 ± 0.05 (76.47)	0.04*	68.76
Wheat (*Triticum aestivum* L. var. 'Bryza')	6.23 ± 1.19 (100)	3.74 ± 1.13 (60.03)	2.28*	13.07 ± 2.98 (100)	7.25 ± 2.20 (55.47)	1.67*	0.19 ± 0.04 (100)	0.13 ± 0.03 (68.42)	0.02*	60.03
Blue lupine (*Lupinus angustifolius* L. var. 'Karo')	2.66 ± 0.63 (100)	0.71 ± 0.34 (26.69)	0.48*	6.05 ± 1.76 (100)	3.75 ± 1.06 (61.98)	0.94*	0.57 ± 0.17 (100)	0.46 ± 0.12 (80.70)	0.09*	26.69
Sunflower seeds (*Helianthus annuus*)	8.01 ± 3.11 (100)	0.79 ± 0.13 (9.86)	2.01*	4.05 ± 1.18 (100)	2.21 ± 0.85 (54.57)	0.76*	0.46 ± 0.18 (100)	0.22 ± 0.07 (47.83)	0.08*	9.86
Tomato (*Lycopersicon esculentum* var. 'Faworyt')	12.48 ± 3.05 (100)	1.28 ± 0.37 (10.26)	1.43*	2.25 ± 0.44 (100)	1.43 ± 0.40 (63.56)	0.22*	0.1 ± 0.04 (100)	0.09 ± 0.05 (90.00)	n.d.	10.26
Alfalfa (*Medicago sativa* L.)	4.33 ± 0.92 (100)	0.66 ± 0.25 (15.24)	1.58*	2.71 ± 0.79 (100)	1.06 ± 0.55 (39.11)	0.68*	0.02 ± 0.01 (100)	0.01 ± 0.005 (50.00)	n.d.	15.24
Cuckooflower (*Lepidium sativum*)	11.75 ± 2.30 (100)	2.67 ± 0.68 (22.72)	2.72*	2.62 ± 0.73 (100)	2.41 ± 0.74 (91.98)	n.d.	0.03 ± 0.02 (100)	0.01 ± 0.01 (33.33)	0.01*	22.72
Lentil (*Lens culinaris* Medik.)	3.29 ± 1.81 (100)	0.62 ± 0.24 (18,84)	0.94*	2.13 ± 0.66 (100)	2.18 ± 0.68 (102.35)	n.d.	0.12 ± 0.05 (100)	0.07 ± 0.04 (58.33)	0.03*	18.84

$LSD_{0.05}$– less significant difference $a < 0.05$; ± SD – standard deviation; * The significance of differences at the level of $a < 0.05$; n.d.- nonsignificant difference;

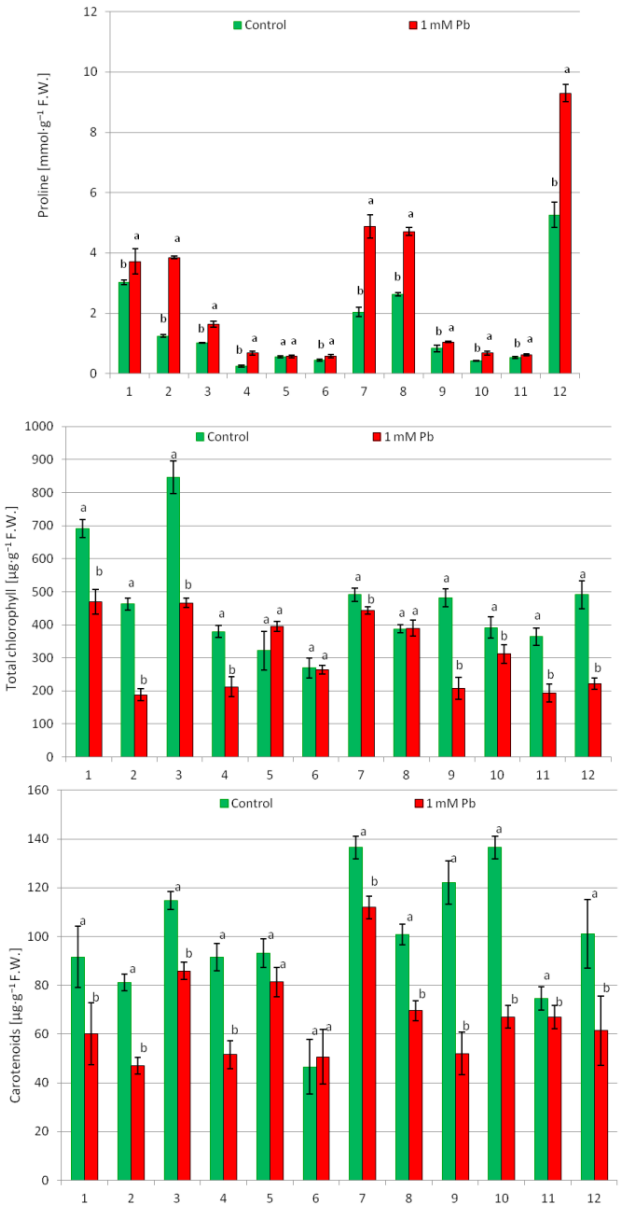

Figure 2. Summary of physiological parameters in the leaves of seedlings of various species of plants growing under stress conditions induced with 1 mM Pb salt. The error bars indicate mean ± SD (n = 3), followed by different letter are statistically significant at $\alpha < 0.05$ levels. F.W. - fresh weight; Numbers 1–12 are: 1 -pumpkin,2 - radish, 3 - cucumber, 4 - barley, 5 - rye ordinary, 6 - wheat, 7 - blue lupine, 8 - sunflower, 9 - tomato, 10 - alfalfa, 11 - cuckooflower and 12 - lentil;

side, in pumpkin, radish, cress and lentil, no statistically significant difference was observed in the seedling growth of plants originating from control and control with 1 mM Pb. The inhibitory effect was more pronounced on root length than on shoot length (Table 1). Sharma and Dubey [2005] reported that roots assimilate Pb better than leaves; therefore, symptoms of toxicity are more enhanced in underground than overground organs. This dependence was also confirmed by Grzesiuk *et al*., [2011], who studied sensitivity to Pb of two varieties of buckwheat. Inhibition of root growth and seedling to Pb was previously noticed in various plants [Shafiq *et al*. 2008, Wang *et al*. 2011, Malar *et al*. 2014]. But, Grzesiuk *et al*. [2011] found that low concentration of Pb (0.01 mM) in roots had a stimulatory effect on their growth; although, reduction of their growth by 60% occurred at 1 mM of Pb. Reduced growth of plants could be related to lower number of cell divisions in zone of cell division [Eun *et al*. 2000]. Whereas Jiang and Liu [2010] observed that exposure of *Allium sativum* roots to Pb for 72 hours induced ultrastructural changes, that is: loss of cristae,

mitochondrial swelling, dictyosomes, endoplasmic reticulum vacuolisation and impairment into lamellar organisation of the chloroplast.

In our studies, a significant decrease in fresh biomass ($p \leq 0.05$) was observed in plants growing with concentration of Pb ranging from 8.4 to 66.7% compared to the control (Table 1). Similar decrease in fresh biomass was also found in stress conditions caused by Pb in *Chlorophytum comosum* [Wang *et al*. 2011] and *Eichhornia crassipes* [Malar *et al*. 2014].

TI is an important indicator that reflects the heavy metal tolerance of plants [Yan *et al*. 1997]. After 10 days of exposure to Pb, the TI value in the studied plant species was low that means about high sensitivity of the test plants to Pb (Table 1). The only plants were TI values were higher were recorded in pumpkin, rye and wheat and they were 86.06, 68.76 and 60.03%, respectively. In studies carried out by Shaikh *et al*. [2013], TI was decreasing along with the increasing concentrations of Cr, Cd, Mn and Zn in wheat.

With the absorption and accumulation of heavy metal, the plant undergoes many physiological changes [Liu *et al*. 2009].

Literature data shows that proline has a positive impact to plant water management as well a pivotal role in plant response to metal presence, which is probably related to antioxidative properties, role of chelating metals and ability of proline to protect enzymes [Özturk and Demir 2002]. In our study, concentration of proline significantly increased in the test plants that obtained a dose of Pb at a concentration ranging from 25 to 208% (Figure 2). Similar response to Pb treatment was previously noticed in various plants [Zengin and Munzuroglu 2005, Awaad *et al*. 2010].

Chlorosis of leaves is one of the physiological symptoms of Pb action on plants. Chlorosis is caused by inhibition of synthesis of photosynthetic pigments [Sharma and Dubey 2005]. Myśliwa-Kurdziel and Strzałka [2002] reported that ð-aminolevulinic acid dehydratase, which is involved in the chlorophyll biosynthetic pathway, is very sensitive to the presence of heavy metals.

Pb treatments showed a statistically significant decrease ($p \leq 0.05$) on photosynthetic pigments - total chlorophyll content and carotenoids of plants leaves compared to control (Figure 2).

In studies by Malar *et al*. [2014], where plants were treated to 1000 mg·L^{-1} of Pb, the reduction in chlorophyll a, b and carotenoid contents was 55, 67 and 55%, respectively, compared to the control.

4. CONCLUSIONS

Based on the obtained results, it was assumed that the concentration of Pb applied in this study inhibited seed germination and growth of roots and shoots, fresh biomass of plants and the content of assimilation pigments and increased the content of proline in 10-day-old seedlings in the studied plant species.

However, reaction of the test plants to Pb was different. Within the studied plants, there were species that in lower degree reacted to stress conditions. In these plants, small or no statistically significant difference was found in morphological and physiological parameters between the control plants and plants exposed to Pb. Among the 12 studied plants, 3 species (pumpkin, rye and whet) presented high tolerance to Pb.

Among the studied plants, there were also species that significantly reacted to stress conditions. This reaction was reflected by statistical significant decrease in average morphological and physiological parameters: decrease in the content of assimilation pigments and increase in proline level compared to the control plants. The most sensitive to Pb exposure were radish, barley, tomato and alfalfa.

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The Effect of Use of the Biologically Active Substances in Alleviating the Stress Caused by Lead in Barley Seedling on the Basis of Biochemical and Physiological Parameters

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ABSTRACT

Plants are constantly exposed to a variety of stressors during their lives. One of such stressors is contamination of the environment with heavy metals. Lead is one of highly toxic metals and it significantly inhibits normal plant growth. The study aimed at assessing the degree of relieving the stress caused by 1 mM $Pb(NO_3)_2$ via different biologically active substances (AsA, GSH, PP, α-Toc, SA) on the basis of the measurement of morphological (root length, coleoptile length, fresh weight), biochemical (Pro, MDA, CAT, POX) and physiological (Chl a+b, Car) traits in 10-day leaves of spring barley of the cultivar Eunova under laboratory conditions. Pb-stress reduced the fresh weight, root length and coleoptiles of the barley tested. Lead increased lipid peroxidation and Pro content, enhanced CAT and POX activity, and significantly suppressed the photosynthetic pigments content. Among the substances used in the experiment, PP, α-Toc and GSH generally relieved the toxic effect of lead to the barley seedlings to the greatest degree.

Keywords: barley, biologically active substances, catalase, lead, malondialdehyde, peroxidase, pigments, proline.

INTRODUCTION

An increase in the addition of heavy metals in the environment causes losses to agricultural crops. Lead is a toxic element, easily assimilated by plants and accumulated in various parts thereof. Plant responses to environmental stresses are complex, limiting plant growth and yield by affecting morphological, physiological and biochemical parameters (Sharma and Dubey, 2005; Sędzik et al., 2015). One of the biochemical changes occurring when plants are subjected to heavy metals, including Pb, is the production of reactive oxygen species (ROS) (Shahid et al., 2014). Enhanced production of ROS in plants during stress can enhance oxidative processes (such as: membrane lipid peroxidation, protein oxidation), enzyme inhibition and DNA and RNA damage (Asada, 2006). To control ROS levels and protect cells from damage, plants produce numerous efficient defense mechanisms, known as the antioxidant defense system. This system consists of low-molecular weight antioxidant compounds (ascorbic acid (AsA), reduced glutathione (GSH), carotenoids, tocopherols) and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX) and enzymes of the ascorbateglutathione cycle (AsA-GSH) (Mishra et al., 2009).

Effective solutions are necessary to increase the tolerance of plants to environmental stresses, including heavy metals. This can be achieved through the use of biologically active substances. Under stress conditions in plants, endogenous levels of these substances are low, which can be counteracted by their exogenous application, which is an environmentally friendly approach. Such exogenous application of plant

non-enzymatic antioxidants, plant growth regulators, plant extracts with secondary metabolites can mitigate the adverse effects of heavy metals on growth, yield as well as biochemical and physiological processes in plants (Chen et al., 2007; Jazi et al., 2011; Al-Hakimi and Hamada, 2011; Son et al., 2014; Jazi and Oregani, 2014).

Vitamin C [ascorbic acid (AsA)] is an antioxidant molecule and a crucial substrate for the detoxification of ROS, molecule essential for the regulation of key physio-biochemical processes in plants (Ishikawa et al., 2006; Moghadam, 2016). Studies by several authors have shown that AsA is effective in improving the plant stress tolerance. It can improve the tolerance to abiotic stresses by enhancing plant growth; it is also involved in the regulation of photosynthesis (protection of photosynthetic pigments), transpiration, protection of lipids, proteins, and enzymes (Al-Hakimi and Hamada, 2011; Venkatesh and Park, 2014).

Glutathione (GSH) is a tripeptide composed of glutamate, cysteine, and glycine. As a non-enzymatic antioxidant, GSH protects plants from oxidative damage caused by stress factors. It is present in all plant cells. It is involved in detoxification of ROS, as well as heavy metals. Glutathione is a substrate for the synthesis of phytochelatins, which are involved in the detoxification of heavy metal ions (Foyer and Noctor 2005, Sharma and Dietz 2006). Nicotinamide (vitamin PP) is a water-soluble vitamin. It is part of the NADH and NADPH coenzymes, which are involved in many enzymatic oxidations, i.e. reduction reactions in cells. It participates in the repair of damage caused by ROS (Abdelhamid et al., 2013). α-Tocopherol (α-Toc, vitamin E) is low molecular a lipid-soluble antioxidant. It is synthesized in chloroplasts by all plants. α-Toc is a key molecule for the detoxification of ROS and protects against oxidation damage. Studies by numerous authors have shown that tocopherol proves to be effective in improving the plant tolerance to various stress environments (Kumar et al., 2012; Sadak and Dawood, 2014).

Salicylic acid (SA) belongs to a group of phenolic compounds. It is a phytohormone with a signaling function in plants (Miura and Tada, 2014). SA is involved in the regulation of important physiological and biochemical processes such as seed germination, growth, development, ion uptake and transport, membrane permeability, photosynthesis, and amino acid metabolism. This acid induces the production of certain stress proteins, thereby participating in plant defense against abiotic and biotic stresses (Khan et al, 2012; Naser et al, 2014; Miura and Tada, 2014).

Barley is one of the basic cereals cultivated in Europe. The high barley yielding potential causes the species to increase its popularity in Europe, and its cultivation area is constantly extended. Barley is grown mainly for animal feed and malt for brewing (Fischbeck, 2003). The study of Sędzik et al. (2015) determined that barley is sensitive to the effect of lead. The experiment investigated whether the addition of exogenous application of biologically active substances (BAS) such as ascorbic acid, glutathione, nicotinamide, α-tocopherol and salicylic acid alleviates the harmful effect of 1 mM lead nitrate stress on barley seedlings.

MATERIAL AND METHODS

The research was carried out under laboratory conditions at the Department of Microbiology and Environmental Biochemistry of the West Pomeranian University of Technology in Szczecin (lat. 53°26′17″ N, long. 14°32′32″ E). The plant material consisted of naked seeds of spring barley (*Hordeum vulgare* L.) of the 'Eunova' cultivar. They were purchased from a specialist shop as certified seeds in class (C/1).

The sensitivity to the presence of 1 mM $Pb(NO_3)_2$ and the extent to which its toxicity was mitigated by the biologically active substances: ascorbic acid (1 mM AsA), glutathione (100 μM GSH), nicotinamide (50 μM PP), a-tocopherol (1 mM α-Toc), salicylic acid (1 mM SA) were assessed for barley seeds and seedlings. Seed disinfection was carried out according to the method described by Krupa-Malkiewicz et al. (2018). Firstly, 1 mM $Pb(NO_3)_2$ and BAS were dissolved in water and poured into vessels and then disinfected barley seeds were introduced into the appropriate combinations of the experiment. For treatments: 30 cm^3 of 1 mM $Pb(NO_3)_2$, 30 cm^3 of (BAS) like: AsA, GSH, PP, α-Toc, SA, respectively and their combinations (AsA+Pb, GSH+Pb, PP+Pb, α-Toc+Pb, SA+Pb) were used. The experiment was set up in six replications (six Petri dishes Ø10 cm with 10 seeds for each treatment).

The seed dishes were incubated for 72 hours in the dark at 21°C, then the pre-germinated seeds were transferred to the phytotron where the light intensity was set to 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ and kept for a further 10 days at 25°C. During the experiment, the photoperiod was set to 16:8 hours. After 10

days, morphological parameters (root length, coleoptile and fresh weight of the seedling), biochemical parameters (Proline - Pro, malondialdehyde – MDA, *catalase – CAT and peroxidase – POX activities*) and physiological parameters (concentrations of total chlorophyll – Chl a+b and carotenoids – Car) were measured.

Determination of the MDA content

The content of malondialdehyde (MDA), as a product of lipid peroxidation, was determined with thiobarbituric acid (TBA) according to the method of Sudhakar et al. (2001). The absorbance of the supernatant was measured at 532 nm and 600 nm using a spectrophotometer UV-1800 produced by Shimadzu. The MDA content was expressed in $\mu mol \cdot g^{-1}$ of fresh plant weight (FW).

Determination of the Pro content

Proline content was determined by ninhydrin reaction according to the method of Bates et al. (1973). The mixture obtained by the reaction was then extracted with toluene. In the collected toluene layer, the absorbance of the dyed chromophore against toluene was determined on a spectrophotometer at 520 nm. The Pro content was expressed in $\mu mol \cdot g^{-1}$ of fresh plant weight (FW).

Determination of the catalase and peroxidase activities

The catalase (CAT) activity of [EC 1.11.1.6] was determined spectrophotometrically according to the method of Lück (1963). The CAT activity was expressed as $\mu mol\ H_2O_2 \cdot g^{-1} \cdot min^{-1}$ fresh plant weight (FW). In contrast, total peroxidase activity (POX) [EC 1.11.1.7] was measured spectrophotometrically according to Chance and Maehly (1955). The POX activity was presented as μmol purpurogallin$\cdot g^{-1}$ fresh plant weight (FW).

Determination of pigment content

The Chl a and Chl b contents were determined in fresh weight according to the method presented by Arnon et al. (1956) and modified by Lichtenthaler and Wellburn (1983). The Car content was assessed according to the method of Hager and Meyer-Berthenrath (1966). The results obtained for pigment content were expressed in $\mu g \cdot g^{-1}$ plant fresh plant weight (FW).

Statistical analysis

Statistical analyses were performed using Statistica 13 (TIBCO Software Inc.). The results obtained were analyzed using descriptive statistics (mean, standard deviation) and two-ways ANOVA. The means were compared using Tukey's HSD test with a significance level at $p < 0.05$. The significance was set at $p < 0.05$. Means were used to carry out cluster analysis using Ward's agglomerative method (using Euclidean distances). Created groups were used to perform MDS (MultiDimensional Scaling), stress and bootstrap in R (R Core Team R), package SMACOF (de Leeuw and Mair 2009). In addition, the differences between effects of BAS *per se* have been shown in MDS using Eta^2 and 'raw' effect of interactions BAS×Pb. The data for Eta^2 has been taken from ANOVA, whereas 'raw' effect of interactions BAS×Pb has been calculated using formula presented in Table 1.

RESULTS AND DISCUSSION

Abiotic stresses (heavy metals, salinity, ozone, UV-B radiation, extreme temperatures, or drought) are among the most challenging threats to agricultural system and economics of yield of crop plants. Numerous studies demonstrate, that physiological and biochemical processes are disturbed under the conditions of abiotic stress, which limits the growth and yielding in plants (Sharma and Dubey, 2005; Khan, 2015). In recent years, with the development of modern cultivation methods, increased interest of different types of chemical compounds is observed, which could fulfill different anti-stress functions in a plant, relieving unfavorable abiotic effects in crops.

Effects of BAS on seedling growth parameters under lead stress

An analysis of morphological traits of the barley seedlings allowed observing that the lead salt influenced on average on the reduction of root's and coleoptile's length and fresh weight of the seedlings with respect to the control, by 81.8%, 36.6% and 41.2%, respectively.

The use of exogenous BAS like: GSH, and SA reduced by 0.9 to 10.75% both roots and coleoptiles length in comparison to the control, although not all means were significantly different (Table

Table 1. The influence of BAS on *morphological* parameters, MDA, Pro, Chl a+b, Car contents, CAT and POX activities in barley seedlings exposed to 1 mM $Pb(NO_3)_2$ treatment under laboratory conditions

Parameter	CL [cm]		RL [cm]		FW [g]		MDA [μmol·g^{-1}FW]		Pro [μmol·g^{-1}FW]		Chl a+b [μg·g^{-1}FW]		Car [μg·g^{-1}FW]		CAT [μmol H_2O_2·g^{-1}FW·min^{-1}]		POX [μmol purpurogallin ·g^{-1}FW]	
[1]Control	10.37 ±1.63	a	9.58± 1.69	a	0.27± 0.02	a	22.42 ±0.85	c	0.21± 0.01	c	286.28± 5.3	c	98.81 ±1.73	b	19.8 ±2.94	d	7.09± 2.36	b
[1]Pb	6.57± 0.99	b	1.74± 0.43	b	0.16± 0.03	b	62.04 ±4.32	a	0.54± 0	a	190.4 ±2.76	d	67.9 ±0.98	d	67.66 ±1.63	a	56.48 ±9.12	a
ASC	11.42 ±2.26	a	10.27 ±1.72	a	0.29± 0.04	a	22.58 ±1.86	c	0.23± 0.01	c	325.2 ±3.39	a	105.29 ±1.38	a	26.7 ±1.58	c	14.88 ±2.54	b
ASC+PB	8.33± 1.09	c	2.38± 0.28	b	0.17± 0.03	b	49.3± 1.14	b	0.47± 0.01	b	306.03 ±3.7	b	90.73 ±2.06	c	42.14 ±0.41	b	21.46 ±6.54	b
Mean±SD	9.17± 1.49		5.99± 1.03		0.22± 0.03		39.09 ±2.04		0.36± 0.01		276.98 ±3.79		90.68 ±1.54		39.07 ±1.64		24.97 ±5.14	
[2]Eta^2	0.013		0.000		0.000		0.721		0.838		0.973		0.909		0.966		0.833	
[3](control-ASC) - (Pb - ASC+Pb)	0.701		-0.055		-0.002		-12.903		-0.082		76.711		16.342		-32.432		-42.814	
Control	10.37 ±1.63	a	9.58± 1.69	a	0.27± 0.02	a	22.42 ±0.85	c	0.21± 0.01	d	286.28 ±5.3	b	98.81 ±1.73	b	19.8 ±2.94	d	7.09 ±2.36	c
Pb	6.57± 0.99	c	1.74± 0.43	b	0.16± 0.03	c	62.04 ±4.32	a	0.54± 0	a	190.4 ±2.76	d	67.9 ±0.98	d	67.66 ±1.63	a	56.48 ±9.12	a
GSH	9.63± 0.98	a	9.5± 1.45	a	0.27± 0.02	a	25.43 ±0.25	c	0.25± 0	c	322.99 ±8.06	a	113.74 ±2.85	a	27.94 ±3.73	c	16.93 ±2.82	b
GSH+Pb	7.88± 1.86	b	2.22± 0.49	b	0.19± 0.04	b	43.44 ±0.52	b	0.48± 0.02	b	253 ±13.16	c	83.26 ±0.56	c	43.83 ±1.18	b	22.98 ±1.71	b
Mean±SD	8.61± 1.36		5.76± 1.02		0.22± 0.03		38.33 ±1.48		0.37± 0.01		263.17 ±7.32		90.93 ±1.53		39.81 ±2.37		25.87 ±4	
Eta^2	0.120		0.015		0.069		0.899		0.867		0.478		0.005		0.935		0.876	
(control-GSH) - (Pb - GSH+Pb)	2.044		0.553		0.029		-21.613		-0.097		25.882		0.425		-31.976		-43.350	
Control	10.37 ±1.63	a	9.58± 1.69	b	0.27± 0.02	b	22.42 ±0.85	c	0.21± 0.01	c	286.28 ±5.3	b	98.81 ±1.73	b	19.8 ±2.94	d	7.09 ±2.36	c
Pb	6.57± 0.99	c	1.74± 0.43	c	0.16± 0.03	d	62.04 ±4.32	a	0.54± 0	a	190.4 ±2.76	c	67.9 ±0.98	c	67.66 ±1.63	a	56.48 ±9.12	a
PP	10.69 ±1.86	a	10.75 ±1.31	a	0.31± 0.07	a	22.96 ±2.23	c	0.22± 0.01	c	360.28 ±2.62	a	113.65 ±5.13	a	31.19 ±3.18	c	9.31 ±1.62	c
PP+Pb	8.59± 2.02	b	2.4± 0.39	c	0.21± 0.02	c	42.37 ±0.65	b	0.42± 0.01	b	291.1± 12.75	b	91.36 ±3.56	b	44.02 ±1.85	b	22.42 ±2.41	b
Mean±SD	9.06± 1.62		6.12± 0.96		0.24± 0.03		37.45 ±2.01		0.35± 0.01		282.02 ±5.86		92.93 ±2.85		40.67 ±2.4		23.82 ±3.88	
Eta^2	0.064		0.014		0.002		0.861		0.930		0.566		0.393		0.949		0.835	
(control-PP) - (Pb - PP+Pb)	1.700		-0.514		0.007		-20.215		-0.123		26.699		8.613		-35.037		-36.285	
control	10.37 ±1.63	b	9.58± 1.69	b	0.27± 0.02	b	22.42 ±0.85	c	0.21± 0.01	c	286.28 ±5.3	a	98.81 ±1.73	ab	19.8 ±2.94	d	7.09 ±2.36	b
Pb	6.57± 0.99	d	1.74± 0.43	c	0.16± 0.03	d	62.04 ±4.32	a	0.54± 0	a	190.4 ±2.76	c	67.9 ±0.98	c	67.66 ±1.63	a	56.48 ±9.12	a
α-Toc	11.47 ±1.05	a	10.65 ±1.81	a	0.31± 0.05	a	24.62 ±0.57	c	0.22± 0.02	c	288.12± 2.92	a	107.1 ±13.36	a	32.95 ±2.7	c	12.55 ±0.53	b
α-Toc+Pb	8.36± 1.03	c	2.08± 0.36	c	0.2± 0.03	c	42.53 ±0.81	b	0.44± 0.01	b	250.1 ±17.1	b	81.41 ±5.64	bc	43.5 ±1	b	15.89 ±2.94	b
Mean±SD	9.19± 1.18		6.01± 1.07		0.24± 0.03		37.9 ±1.64		0.35± 0.01		253.73 ±7.02		88.8 ±5.43		40.98 ±2.07		23± 3.74	
Eta^2	0.021		0.022		0.001		0.897		0.867		0.789		0.046		0.964		0.891	
(control-α-Toc) - (Pb-α-Toc +Pb)	0.697		-0.741		0.005		-21.720		-0.116		57.861		5.242		-37.316		-46.053	
Control	10.37 ±1.63	a	9.58± 1.69	a	0.27± 0.02	a	22.42 ±0.85	c	0.21± 0.01	c	286.28 ±5.3	b	98.81 ±1.73	ab	19.8 ±2.94	d	7.09 ±2.36	c
Pb	6.57± 0.99	b	1.74 ±0.43	b	0.16± 0.03	c	62.04 ±4.32	a	0.54± 0	a	190.4 ±2.76	d	67.9 ±0.98	c	67.66 ±1.63	a	56.48 ±9.12	a
SA	9.25± 1.34	a	9.36± 1.01	a	0.25± 0.02	b	25.11 ±1.62	c	0.27± 0.01	b	355.43 ±2.65	a	105.33 ±5.85	a	33.02 ±1.88	c	9.92 ±1.64	bc
SA+PB	7.16± 1.6	b	1.79± 0.29	b	0.17± 0.03	c	34.19 ±1.28	b	0.51± 0.02	a	266.01 ±7	c	93.63 ±5.72	b	40.05 ±1.41	b	19.94 ±1.9	b
Mean±SD	8.34± 1.39		8.34± 1.39		0.22± 0.02		35.94 ±2.02		0.38± 0.01		274.53 ±4.43		91.42 ±3.57		40.13 ±1.96		23.36 ±3.75	
Eta^2	0.087		0.004		0.122		0.937		0.795		0.146		0.661		0.974		0.859	
(C ontrol-SA) - (Pb - SA+Pb)	1.701		0.257		0.034		-30.538		-0.085		6.456		19.208		-40.833		-39.372	

Note: [1] For the clarity of the interpretation of the results, in the Table 1 it has been repeated means for the dependent variables (traits investigated) obtained in the tests for the control and Pb; [2]Eta^2 - values from teh ANOVA analysis. They describe the percentage of the variance explained by the BAS×Pb interaction for each described dependent variable. They were used in the study for the MDS analysis. [3]the ‚raw' effect of variance was calculated for each BAS according to the formula (control - AsA) - (Pb - AsA+Pb), and the differences were used in the MDS analysis; a-d - homogeneous groups.

1). The use of AsC, PP and α-Toc had the opposite effect. The use of biologically active substances stimulated the seedling fresh weight from 6.2 to 14.5% and stimulated by 3.1 to 12.3% both roots and coleoptiles length with regards to the control (Table 1). On the other hand, the use of combination BAS with lead salt caused a reduction in the toxic effect of lead ions on the formation of morphological parameters of the barley seedlings. In this case, the highest "relieving" role on the inhibition of the root and coleoptile length and fresh weight of the seedlings was determined after the use of nicotinamide (Table 1). Such effect of the used nicotinamide may stem from the fact that it fulfills numerous functions within plants. Nicotinamide influences the induction and regulation of the metabolic response in a plant organism exposed to a stress factor and is a component and manifestation of the defense metabolism in plants (Berglund and Ohlsson, 1995). In addition, the compound has been shown to have a positive effect on many physiological processes, such as the biosynthesis of enzymes, nucleic acids and proteins as a growth-regulating factor, in addition to acting as a coenzyme (Hathout, 1995). The results obtained in the present study are in line with the results reported by other authors (Dawood et al., 2019; Mohamed et al., 2020; Sadak et al., 2010). They demonstrated that the addition of exogenous BAS caused a growth of the roots, coleoptiles and plant fresh weight stressed by different abiotic factors. Reports on negative effects resulting from the use of SA on the growth of plants can be found in the scientific literature. The study conducted by Klocek and Mioduszewska (2001) determined the influence of SA on the length of the shoots, as well as the number of leaves, roots, and tubers formed in a potato. According to Basra et al. (2007) the inhibition of plant growth under the influence of SA is observed in its higher concentrations. These authors suggest that this may stem from the restriction in the absorption of nutrients due to the disturbances of membranes integrity. On the other hand, the study of Jazi et al. (2011) on the alleviating of the negative effect of $Pb(NO_3)_2$ showed that the most efficient SA dose turned out to be 10 µM. A similar limitation in growth efficiency was found in goji in response to 1 mM $Pb(NO_3)_2$ (Krupa-Małkiewicz et al., 2018). These authors reported that in the presence of 1 mM ascorbic acid in MS medium with 1 mM $Pb(NO_3)_2$ the shoot and root lengths of goji were enhanced by 31% and 74.5%, respectively, compared to lead-treated explants. In the study of Al-Hakimi and Hamada (2011), the negative effects of Cu toxicity to the growth of roots and shoots were partially alleviated by treatment of plants with ascorbic acid solutions, thiamine (vitamin B_1) and salicylic acid.

Effects of BAS on the MDA content in leaves of barley under lead stress

Lead stress significantly increased the MDA content (by 176.7%) in coleoptiles with regards to the control (Table 1). Application of lead with combination of BAS effectively alleviated lipid peroxidation as reduced MDA content in the barley leaves. All means (Pb *versus* BAS+Pb) were significantly different (Table 1). The greatest decrease of MDA (by 44.9%) content in barley seedlings was observed after the use of SA, the lowest (20.5%) – after the use of AsA. Similar results were obtained by Khattab (2007) on canola, and Cao et al. (2013) on rice seedlings. In contrast, in a study by Krupa-Małkiewicz et al. (2018), it was observed that the presence of 1 mM AsA with 1 mM $Pb(NO_3)_2$ in MS medium had an inhibitory effect on the MDA content by 4% in goji seedlings compared to Pb treatment. However, 1 mM ASA induced MDA accumulation in goji seedlings by 24% when compared to the control. Lokhande et al. (2011) and Cao et al. (2013) suggested that the higher MDA concentration in plant tissues may be responsible for the reduction in membrane lipid peroxidation, which is related to high membrane oxidative damage and therefore higher H_2O_2 production. In contrast, Metwally et al. (2003) reported an increase in MDA of about 50% after the exposure to Cd in roots of SA-free controls. The effect of SA on lipid peroxidation was not due to a reduction in Cd accumulation in roots and shoots.

Effects of BAS on Pro in leaves of barley seedlings under lead stress

Application of lead salt significantly increased the proline content by an average of 153% compared to the control (Table 1). Although for all applied BAS the proline content was higher if compared to the control (H_2O) on average by 7.9% (AsA), 18.0% (GSH), 3.0% (PP), 5.3% (α-Toc) and 27.7% for SA, only for GSH and SA these differences were described as significant. The application of BAS in the Pb stressed plants significantly reduced the proline content in coleoptiles of

the tested seedlings if compared to the Pb treated seedlings on average by 12.7% (AsA), 10.9% (GSH), 21.7% (PP), 18.4% (α-Toc) and only 5% for SA. The most beneficial effect of minimizing lead-induced stress was found when AsA, PP and α-Toc were applied, respectively. Pavliková et al. (2008) showed that the formation of large amounts of proline at high heavy metal concentrations leads to an increase in the glutamate kinase activity. This gives rise to an increase in the concentration of glutamic acid required for the synthesis of glutathione and phytochelatins in plant cells. Thus, the ability of plants to synthesize large amounts of proline after exposure to Pb stress suggests the ability to tolerate this element (Ozturk and Demir 2002). Pro is an indicator of oxidative stress. It accumulates in plant tissues when exposed to many environmental factors (Zhu et al., 2008) as well as has chelating and antioxidant properties, protecting enzymes from the denaturing effects of ROS. By capturing singlet oxygen, it protects CAT, POX, and polyphenol oxidase, among others (Verbruggen and Hermans 2008).

Effects of BAS on the photosynthetic pigments content in leaves of barley under lead stress

Application of Pb salt in growth media significantly suppressed the photosynthetic pigments content (total chlorophyll and carotenoids) of tested barley seedlings (Table 1). The Pb-induced stress decreased the content of both total chlorophyll and carotenoid, by 33.5 and 31.3% in comparison to the control. However, it was observed that BAS application had a positive effect on the tested traits in comparison to the control. The greatest increase of the total chlorophyll and carotenoids content was determined after the use of SA, by 24.1% and 6.6% and PP by 25.8 and 15.0%, respectively.

The use of all exogenous biologically active substances in combination with Pb significantly reduced the toxicity of lead ions in comparison to the level of photosynthetic pigment content in the seedlings treated only with 1 mM $Pb(NO_3)_2$ (Table 1). According to Chen et al. (2007), Pb inhibits the synthesis and even increases the degradation of chlorophyll, resulting in impaired uptake of essential elements such as Mg and Fe by plants. In their study, they used exogenous SA pretreatment on young rice seedlings. The obtained result showed that SA could enhance the chlorophyll content under Pb stress significantly higher compared to the control. Similar results were obtained by Matewaly et al. (2003) on the influence of the stress caused by the effect of Cd on barley seedlings. In turn, Krupa-Małkiewicz et al. (2018) studied the exposure the goji seedlings to 1 mM $Pb(NO_3)_2$ and observed marked reduction on the contents of chlorophylls a and b and carotenoid, by 21%, 51% and 54%, respectively, with respect to the control. Addition of AsA to MS medium significantly mitigated the negative effect of Pb-stress factor. Khattab (2007) to alleviate saline stress in canola used GSH and Poly (A) (poliadenylic acid) as biologically active substances. According to many authors (Zechmann et al., 2008; Noctor et al., 2012) glutathione shows effects in various cellular functions in biosynthetic pathways, sulfur transport, gene expression, eliminates the reactive oxygen radicals (ROS), and resistance to biotic and abiotic stresses. Glutathione also has a protective function for the plant in forming conjugates with xenobiotics, and acts as a precursor for the synthesis of phytochelatins, which are involved in the detoxification of heavy metals (Cobbett and Goldsbrough, 2002). The canola seeds treated with GSH and Poly (A) significantly increased the contents of chlorophylls a and b. The ameliorating effects of glutathione may be due to their protective role in salinity tolerance by maintaining the redox status. In turn, Abdelhamid et al. (2013) showed that foliar treatment of nicotinamide on faba bean plant in two concentrations (200 and 400 mg) alleviated the effect on the contents of chlorophyll a, chlorophyll b and total pigments. In order to alleviate the effects of temperature stress in corn, Ahmad et al. (2014) used exogenous AsA, SA and H_2O_2. The chlorophyll b contents were increased with exogenous application of AsA, SA and H_2O_2 in maize. This increase in the chlorophyll b content might be due to enhancement in antioxidant production at low temperature which may have protected chlorophyll from degradation.

Effects of BAS on enzyme activities in leaves of barley seedlings under lead stress

The use of lead salt had a significant influence on increase of antioxidant enzyme activities: CAT and POX by mean from 241.7% to 696.6% with respect to the control (Table 1). The increase of the activities of the above-mentioned enzymes in coleoptiles was also observed after the addition

of BAS. The increase for CAT was in the range from 34.9% to 66.8% and for POX from 16.4% to 110%, respectively. The use of BAS influenced the reduction of the activities of the tested enzymes in the Pb-stressed plants, in comparison to the test combination with lead only (Table 1). In the CAT case, the significantly decreased activity of the enzyme was observed for all combinations at a similar level. The use of combination of α-Toc+Pb was most favorable for POX, decreasing the activity of the enzyme by 71.86%. According to Krieger-Liszkay and Trebst (2006), Maeda and DellaPenna (2007) α-tocopherol is an antioxidant that has been demonstrated to deactivate reactive oxygen species from photosynthesis. It also scavenges peroxyl radicals in thylakoid membranes, thereby preventing lipid peroxidation. In turn, Chen et al. (2007) indicated that SA pretreatment in the absence of Pb was the most favorable for decreased CAT activity in rice seedlings. Rucińska-Sobkowiak (2010) indicated that the reports on the changes of antioxidant enzymes under the effect of environmental stresses differ. As reported by Dey et al. (2007), the reason for such different reactions of enzymes under similar stress conditions, may consist in not entirely identical experimental conditions. CAT, POX, SOD are significant antioxidant enzymes, which function in the cells; they are important in order to prevent the excess reactive oxygen species accumulation (Passardi et al., 2005). Hydrogen peroxide is removed by CAT and POX, among others (Cui and Zhao, 2011). The increase of activity of these enzymes indicates oxidative stress in the cells. A significant decrease of the activity of SOD, CAT and POX in plants, as provided by Dey et al. (2007) indicates the weakening of the scavenging systems for the reactive oxygen species, which are found during the effect of a stressor. These authors believe that the decrease of enzyme activity may be caused by enzyme inhibition. These proteins are sensitive to numerous factors. According to Rucińska-Sobkowiak (2010) as well as Cui and Zhao **(2011)** such different reactions in the activity of enzymes (increase, decrease or lack of changes) depend on the plant species, the treatments used, its concentration and the exposure time. The range of plant response to stress differs within one species; a variable tolerance to the same factor is exhibited (Malik et al., 2010).

Ward's agglomerative method was used to group treatments according to the morphological, biochemical and physiological parameters in order to reveal the effective influence of Pb and BAS on seedlings barley growth (Fig. 1). Treatments were clustered in six discrete groups (a–f). Separate groups were formed by the independent variables (control and Pb, respectively) and SA+Pb (Fig. 1). The other two groups included, respectively, BAS – added to investigate the effect *per se* on the

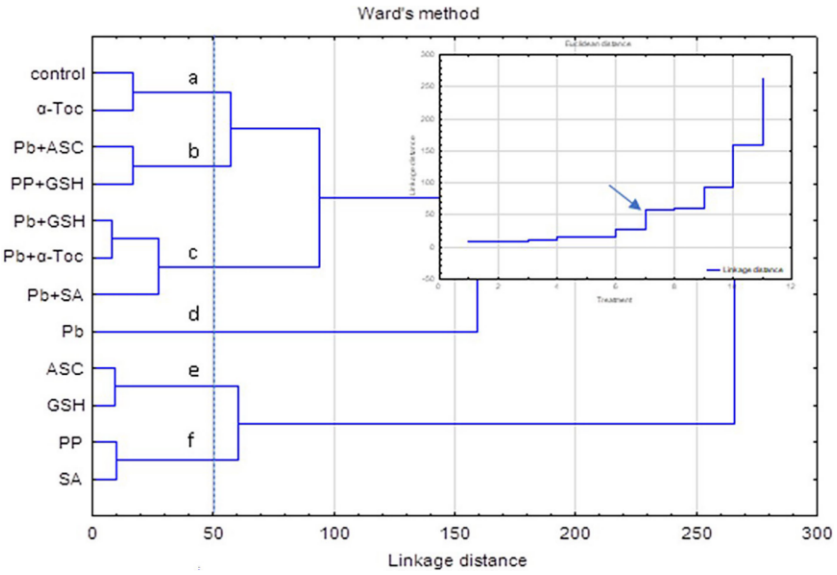

Fig. 1. Dendrogram of cluster analyses of Ward's method of dependent variables determined for seedlings of barley cv. Eunova after using twelve treatments. The vertical lines indicate the cuts-off used to form the five groups (a–f)

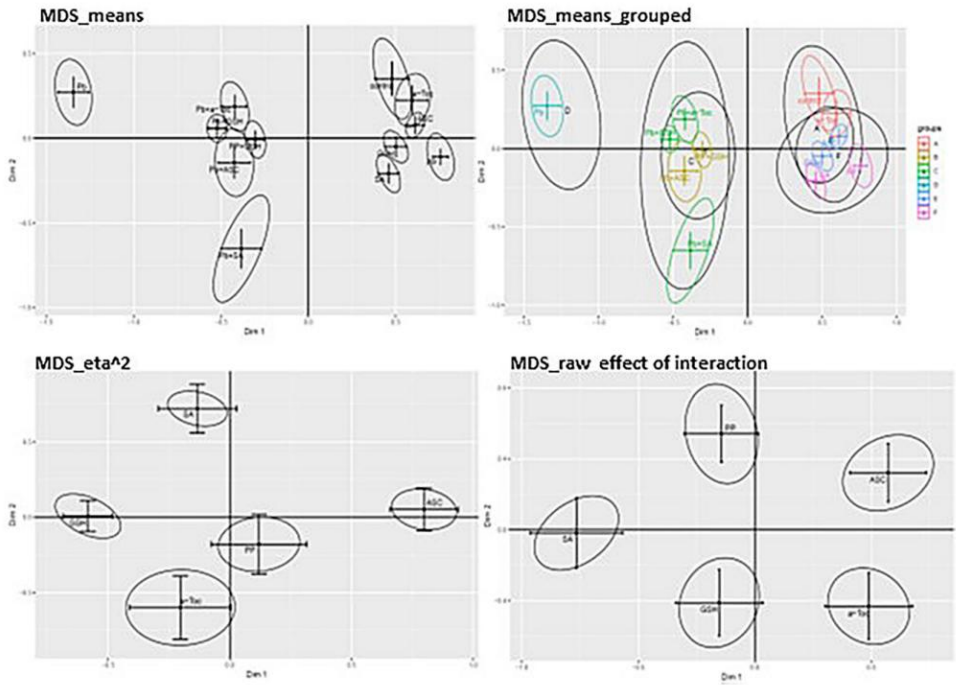

Fig. 2. Two-dimensional plots of MDS performed for treatment datasets using Euclidean distance matrix

studied seedling traits and BAS+Pb to investigate their ability to minimize the stress caused by the presence of lead salts in the solution. It has been shown that BAS inhibits the toxic effect of lead to varying degrees if selected morphological, physiological and biochemical parameters are analyzed (groups b and c). It should be added that Ward's agglomerative method was used by Manschadi et al. (2008) to group wheat genotypes differing in tolerances to drought in terms of growth angle and seminal root number. The effect of Ward's grouping has been used in MDS (Fig. 2). Clearly different coordinates were observed in both graphs for the five different groups. Distinct groups were formed by BAS and BAS+Pb. Others were control, PB and SA+Pb. The splits observed in the two-dimensional spaces of MDS clearly reflect the effect of BAS in alleviating the abiotic stress induced by lead salts.

To better visualize the differences in the interaction of various BASs with Pb used in the experiment, MDS was performed for the Eta^2 values generated in the ANOVA analyses for the interactions described for each BAS and the investigated dependent variables. The distribution of points (individual BAS) in different places of the two-dimensional MDS space suggests differences in the influence of individual BASs on minimizing the negative impact on the toxic effects of Pb. Similarly, for individual BASs, the 2D MDS space is presented after the analysis of the value mapping 'raw' inteaction BAS×Pb variance effect (Fig. 2).

CONCLUSIONS

Lead salts negatively affect the determined morphological, biochemical and physiological parameters in 10-day-old spring barley seedlings. Of the BAS tested, the best effects in mitigating the negative effects of Pb stress were shown by: PP, α-Toc and GSH. In contrast, the lowest were determined for SA. Changes in the values of Pb-induced abiotic stress indicators, such as MDA, Pro, Chl a+b, Car, CAT and POX, reflect the stress status of the plant. They allow seeing and expressing the beneficial effect of BAS in minimizing it.

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ORIGINAL PAPER

EFFECT OF NICOTINAMIDE IN ALLEVIATING STRESS CAUSED BY LEAD IN SPRING BARLEY SEEDLING*

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Abstract

Throughout their lives plants are constantly exposed to abiotic stress. Lead is a highly toxic element and has not yet been shown to have a positive effect on plants. This element causes inhibition of physiological and biochemical processes in plants. Exogenous application of vitamins to plants has been successfully used to minimize the adverse effects of abiotic stresses on plant growth, biochemical and physiological processes. The objective of the present study was to examine whether the adverse effects of 0.5 - 2.0 mM lead nitrate stress on plants could be mitigated by exogenous application of 25 - 100 µM nicotinamide (vitaminum PP). The study material consisted untreated seeds of spring barley (*Hordeum vulgare* var. Eunova). The tolerance to Pb stress was evaluated by measuring morphological traits (root and shoot length, plant fresh weight), biochemical and physiological parameters (malondialdehyde and proline content, catalase activity, total chlorophyll and carotenoids content) of 10-day-old seedlings originated from embryos cultured on the MS which contained lead and exposed to nitrate stress alone or with nicotinamide. All results, when compared to the control, showed that the higher concentration (2.0 mM) of $Pb(NO_3)_2$ had the most remarkable effect on the measured parameters. The reduction of tested parameters in barley seedlings is indicated. The addition of nicotinamide to the MS medium alleviated the adverse effect of $Pb(NO_3)_2$ stress on plant growth and selected biochemical parameters – MDA (malondialdehyd) and proline contents and CAT (catalase activity). Nicotinamide at 50 and 100 µM gives the best effect.

Keywords: abiotic stress, catalase, lead, malondialdehyde, morphological traits, pigments, proline.

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INTRODUCTION

Throughout their life cycle, plants are subjected to various types of environmental stresses, which include salinity, water deficit, temperature extremes, toxic metal ion concentration and UV radiations (Hayat et al. 2012). High levels of lead in the plant tissues cause the production of reactive oxygen species (ROS), which change the structure and permeability of the membranes (Sharma, Dubey 2005, Najeeb et al. 2014). Formation of aldehydes, especially malondialdehyde (MDA), is an important indicator of the damage in the membranes caused by ROS (Zielinski, Portner 2000). The level of MDA is now being exploited as a biomarker of oxidative stress (Otitoloju, Olagoke 2011, Smolik et al. 2013).

Proline plays three major roles during stress, i.e., as a metal chelator, an antioxidative defense molecule and a signaling molecule (Hayat et al. 2012). To scavenge high ROS levels, an efficient system of non-enzymatic and enzymatic antioxidants is involved (Gill, Tuteja 2010). Stress factors, such as heavy metals, can also modulate the activities of antioxidative enzymes. Catalases are highly active enzymes that do not require cellular reductants as they primarily catalyse a dismutase reaction (Gill, Tuteja 2010, Mhamdi et al. 2010).

Vitamins could be considered as bio-regulator compounds which in relatively low concentrations exert profound influences on plant growth regulating factors that influence many physiological processes, such as synthesis of enzymes, act as co-enzymes and affect plant growth (Reda et al. 2005, Hassanein et al. 2009). Nicotinamide (vitamin PP) is a water-soluble vitamin from the B group. Nicotinamide is a constituent of the pyridine dinucleotide coenzymes NADH and NADPH, which are involved in many enzymatic oxidation-reduction reactions in living cells (Abdelhamid et al. 2013, Azooz et al. 2013). Exogenous application of vitamins to plants has been successfully used to minimize the adverse effects of abiotic stresses on plant growth, biochemistry and physiology processes (Hassanein et al. 2009, Sadak et al. 2010, Rady et al. 2011, Abdelhamid et al. 2013, Chaparzadeh, Chagharlou 2013). According to Athar et al. (2008), exogenous application of vitamins through the rooting medium can increase the endogenous content of vitamins.

Cereals are grasses that play an important role in the human diet (Paznocht et al. 2018). A widely-consumed cereal is barley (*Hordeum vulgare*) because of its ready availability, reasonable cost, and processing properties for products, such as beer, barley teas, soup, and baked products (Kim et al. 2007). Seed germination is the initial event in the life of a plant. This process is initiated by the regulation of enzymatic reactions which activate catabolic and anabolic process in the storage tissue and the embryonic axis. If a single component of these processes is affected, seed germination could be inhibited. Heavy metals influence the plant physiology and result in several nutritional disturbances (Abdelhamid et al. 2013).

The objective of the present study was to examine whether the adverse effects of 0.5 - 2.0 mM lead nitrate stress on barley plants could be mitigated by exogenous application of 25 - 100 µM nicotinamide.

MATERIAL AND METHODS

Material

The study material consisted untreated seeds of spring barley (*Hordeum vulgare* var. Eunova). The tolerance to Pb stress was evaluated by measuring morphological traits (root and shoot length, plant fresh weight), biochemical and physiological parameters (malondialdehyde and proline content, catalase activity, total chlorophyll and carotenoids content) of 10-day-old seedlings originated from embryos cultured on the MS (Murashige, Skoog 1962) medium containing 0.5 - 2.0 mM Pb salt alone or with 25 - 100 µM nicotinamide (vit PP). The content of the testing media was established on the basis of earlier tests (Sędzik et al. 2015). The control medium was MS. The experiment was carried out in 16 combinations: 1) control, 2) 25 µM Vit. PP, 3) 50 µM Vit. PP, 4) 100 µM Vit. PP, 5) 0,5 mM $Pb(NO_3)_2$, 6) 0,5 mM $Pb(NO_3)_2$ + 25 µM Vit. PP, 7) 0,5 mM $Pb(NO_3)_2$ + 50 µM Vit. PP, 8) 0,5 mM $Pb(NO_3)_2$ + 100 µM Vit. PP, 9) 1 mM $Pb(NO_3)_2$, 10) 1 mM $Pb(NO_3)_2$ + 25 µM Vit. PP, 11) 1 mM $Pb(NO_3)_2$ + 50 µM Vit. PP, 12) 1 mM $Pb(NO_3)_2$ + 100 µM Vit. PP, 13) 2 mM $Pb(NO_3)_2$, 14) 2 mM $Pb(NO_3)_2$ + 25 µM Vit. PP, 15) 2 mM $Pb(NO_3)_2$ + 50 µM Vit. PP, 16) 2 mM $Pb(NO_3)_2$ + 100 µM Vit. PP. Each combination of the experiment was represented by 100 embryos. The embryos were dissected from the seeds soaked in 0.5% sulfuric acid for 20 min, rinsed three times in sterile distilled water. Next, the seeds were treated with 7% sodium hypochloride, rinsed for 15 min in sterile distilled water and soaked in water for 24 h. Then, the embryos were excised with a needle, kept in 10% sodium hypochloride for 10 min., rinsed with sterile distilled water and transferred to a proper medium in test tubes (30 cm^3), 4 embryos in a test tube (9 cm x 3.5 cm). The test tubes were covered with aluminium foil and parafilm and held for 10 days in a growth chamber at 24°C, 16 h photoperiod (40 µmol m^{-2} s^{-1}) and 55-60 relative humidity. Morphological and biochemical features of 10-days old barley seedlings were compared with the control. The stages of the experiment are presented in Figure 1.

Determination of proline and malondialdehyde content

The concentration of free proline in barley was measured three times. The proline accumulation was determined according to Bates (1973). The content of malondialdehyde (MDA) in plant tissue was determined by the method described by Sudhakar et al. (2001).

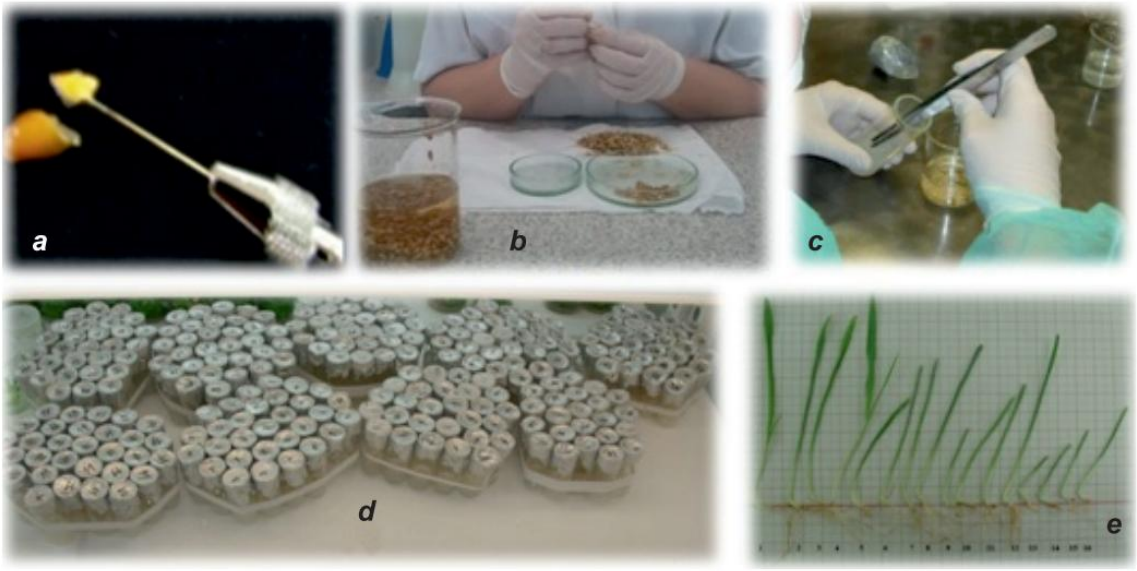

Fig. 1. Preparation steps of spring barley mature embryos under in vitro conditions:
a, *b* – preparation of embryos using a dissecting needle, *c* – desinfection of embryos,
d – embryos on a medium in glass tubes in a growth chamber,
e – ready to measure ten-day-old seedlings

Determination of pigments content

The extraction of leaf pigments was performed with 80% (v/v) acetone. Chlorophyll total (chlorophyll *a*+*b*) and content of carotenoids were determined spectrophotometrically at 663, 645 and 440 nm. The content of chlorophylls was measured according to ARNON et al. (1956) with the modification by LICHTENTHALER, WELLBURN (1983), whereas the content of carotenoids was determined by the method of HAGER, MEYER-BERTHENRATH (1966).

Determination of CAT activity (EC 1.11.1.6)

The method proposed by LUCK (1963) was used to determine the CAT activity. The decrease in absorbance, caused by decomposition of H_2O_2, was monitored continuously at 240 nm for 90 s. One unit of enzyme is the amount necessary to decompose 1 μM H_2O_2 min^{-1}.

Statistical analysis

The significance of differences was determined by means of variance analysis and the Tukey's test, at the level of significance of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Changes in plant growth and development are often affected by various environmental conditions. A common response of plants to heavy metal

stress is growth inhibition (Li et al. 2005, Sędzik et al. 2015). Lead is one of the most dangerous heavy metal pollutants in the environmental that originates from various sources. The main sources of lead are dust and gases from volcanic eruptions, as well as mines, smelters, industry and agriculture. The steadily increasing levels of this metal in the environment inhibit germination of seeds and exert a wide range of adverse effects on the growth and metabolism of plants (Verma, Dubey 2003, Sędzik et al. 2015).

In the present study, the $Pb(NO_3)_2$ treatment inhibited seed germination, root and shoot growth and fresh weight of barley seedlings in comparison to the control (Table 1).

The value of the seed germination index (%) in spring barley seeds under the $Pb(NO_3)_2$ salt stress ranged from 16.77 to 31.40% (Table 1). It was observed, that the lowest germination capacity of barley seeds occurred at the higher Pb salt concentration (2 mM $Pb(NO_3)_2$). Moreover, on the medium with the addition of 0.5 mM $Pb(NO_3)_2$, the addition of PP, regardles of its concentration, did not significantly influence the value of the tested trait. In the case of barley seedlings from MS media supplied with 1.0 and 2.0 mM solution of $Pb(NO_3)_2$, the best efficiency in alleviating the stress effects was

Table 1

Germination index value and summary of morphological characteristics, fresh weight, and the tolerance index of spring barley seedlings growing under stress induced by $Pb(NO_3)_2$ and in the presence of nicotinamide

No.	Treatments	Germination index IG (%)	Root length (cm)	Shoot length (cm)	Fresh weight (g)	Tolerance index (%)
1.	Control	100.00	11.71 ± 1.05	9.36 ± 2.93	0,32 ± 0.01	100.00
2.	25 µM PP	50.99	10.86 ± 3.18	9.16 ± 0.81	0.31 ± 0.01	97.86
3.	50 µM PP	58.68	8.81 ± 0.55	7.11 ± 2.31	0.32 ± 002	75.80
4.	100 µM PP	72.48	9.73 ± 0.71	8.76 ± 1.01	0.33 ± 0.01	93.59
5.	0.5 mM $Pb(NO_3)_2$	21.24	7.23 ± 2.21	6.23 ± 1.76	0.22 ± 0.03	66.19
6.	0.5 mM $Pb(NO3)_2$+25 µM PP	21.74	7.41 ± 1.32	7.01 ± 1.45	0.24 ± 0.08	74.73
7.	0.5 mM $Pb(NO_3)_2$+50 µM PP	23.14	8.21 ± 1.93	7.50 ± 1.41	0.27 ± 0.07	80.07
8.	0.5 mM $Pb(NO_3)_2$+100 µM PP	23.97	8.43 ± 2.72	7.06 ± 2.19	0.29 ± 0.05	75.44
9.	1 mM $Pb(NO_3)_2$	31.40	6.33 ± 1.71	3.84 ± 1.85	0.18 ± 0.14	40.57
10.	1 mM $Pb(NO_3)_2$+25 µM PP	17.93	5.76± 0.55	2.16 ± 0,42	0.18 ± 0.10	23.13
11.	1 mM $Pb(NO_3)_2$+50 µM PP	47.11	7.73 ± 3.12	5.70 ± 1.11	0.21 ± 0.12	60.85
12.	1 mM $Pb(NO_3)_2$+100 µM PP	52.64	8.76 ± 0.20	6.36 ± 1.62	0.23 ± 0.10	67.97
13.	2 mM $Pb(NO_3)_2$	16.78	4.06 ± 1.12	2.03 ± 0.46	0.13 ± 0.01	21.71
14.	2 mM $Pb(NO_3)_2$+25 µM PP	18.18	5.13 ± 0.86	3.03 ± 0.26	0.14 ± 0.03	23.49
15.	2 mM $Pb(NO_3)_2$+50 µM PP	19.01	5.46 ± 0.76	2.3 ± 0.78	0.14 ± 0.01	24.55
16.	2 mM $Pb(NO_3)_2$+100 µM PP	22.89	5.71 ± 0.72	2.76 ± 0.37	0.16 ± 0.04	24.55
	LSD (5%)	-	0,46	0,57	0,03	-

demonstrated by the addition of PP vitamin at the highest concentration – 100 µM. The current literature suggests that seed germination is affected by metals in two ways. Firstly, by their general toxicity, and secondly, by their inhibition of water uptake (Kranner, Colville 2011). Seed germination inhibition by heavy metals has been reported by Verma, Dubey (2003) in rice, Li et al. (2005) in *Arabidopsis thaliana* and Sędzik et al. (2015) in various plant species.

Our results indicate a decrease in vigour (length and weight) of barley seedlings when increasing concentrations of $Pb(NO_3)_2$ in MS medium were tested (Table 1). With 2 mM $Pb(NO_3)_2$ in the MS medium, up to a 65% reduction in root length and 78% reduction in shoot length in comparison with the control group was observed in 10-day-old seedlings. Similarly, up to a 59% decline in plant fresh weight compared with the control was noticed in seedlings at 10-days' growth. Moreover, control or stressed seedlings showed a higher plant fresh weight with than without nicotinamide (Table1). This may indicate that that the presence of nicotinamide in the MS medium supplemented with $Pb(NO_3)_2$ raised the Pb tolerance of barley seedlings, as indicated by IT (%). It was observed that the concentration of nicotinamide did not significantly influence the IT value only when 2.0 mM $Pb(NO_3)_2$ were used. According to many authors (Verma, Dubey 2003, Li et al. 2005, Sędzik et al. 2015), roots assimilate Pb better than leaves. However, leaves differ in their abilities to accumulate Pb depending on age. The role of vitamins in modifying the environmental stresses induced changes in the osmoprotectant content and was also investigated by Sadak et al. (2010), Abdelhamid et al. (2013). According to Berglund, Ohlsson (1995), nicotinamide may be of value in biotechnology for the production of valuable substances as well as for plant protection. This vitamin might act as an activator of protein synthesis through modulation of the activity of enzymes involved in the metabolism of proteins or sugars.

Moreover, nictotinamide can significantlt improved physiological and biochemical parameters (Abdelhamid et al. 2013). According to Verma, Dubey (2003), the heavy metals (Cd, Pb, Al, Zn) are known to produce ROS and induce oxidative stress in certain plant species. MDA is one of the end products that are produced as a result of lipid peroxidation damage by free radical. Heavy metal stress significantly increases MDA (from 27.47 to 32.09 nmol g^{-1} fm), and at 2.0 mM $Pb(NO_3)_2$ the effect was more pronounced (Figure 2). Application of nicotinamide (irrespective of the concentrations) attenuated the effect of heavy metal stress, by decreasing the MDA level in comparison to the control (22.90 nmol g^{-1} fm). Proline accumulation is one of the most frequently reported modifications induced by environmental stresses (Kahlaoui et al. 2018). In this study, the content of proline was significantly increased (1.41 µmol g^{-1} fm) when 2.0 mM $Pb(NO_3)_2$ were used in comparison to the control (1.04 µmol g^{-1} fm). A similar response to a Pb treatment was previously noticed in various plants (Zengin, Munzuroglu, 2005, Awaad et al. 2010, Sędzik et al. 2015). Application of nicotinamide into the medium under

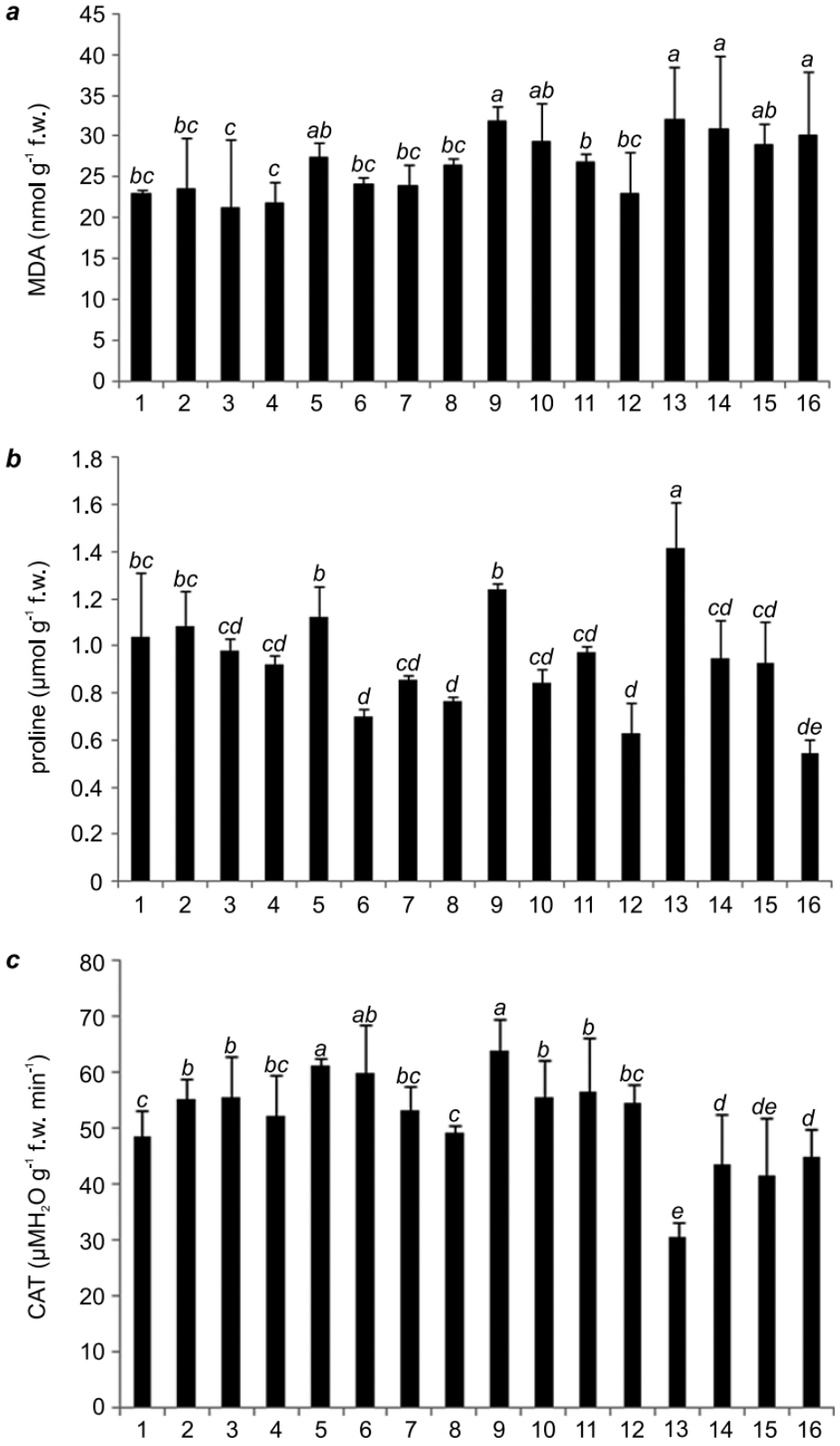

Fig. 2. Summary of biochemical parameters (*a* – MDA, *b* – proline, *c* – CAT activity) in spring barley seedlings growing under stress induced by lead in the presence of nicotinamide; values denoted with the same letters within an experimental variant do not differ statistically ($p < 0{,}05$); no. 1-16 – see Table 1

heavy metal stress condition significantly decreased the proline level in 10-day-old barley seedlings (0.55 - 0.96 µmol g^{-1} fm) (Figure 2). These results are in agreement with the results observed by Azooz et al. (2013), who suggested that most of the vitamins tend to increase the proline content.

In our study, the activity of catalase (CAT) was observed to have increased during the 10-day-long growth of barley seedlings under heavy metal stress (Figure 2). With increasing levels of $Pb(NO_3)_2$ in the MS medium, the activity of CAT peaked (63.87 µM H_2O_2 g^{-1} fm) in comparison to the control (48.71 µM H_2O_2 g^{-1} fm). However, the activity of CAT significantly decreased (41.42 - 59.98 µM H_2O_2 g^{-1} fm) when nicotinamide was used. Moreover, the higher concentration of nicotinamide (2.0 µM) was more effective than the lower one (0.5 µM). The results are in agreement with the ones obtained by Verma, Dubey (2003), who suggested that the activity of catalase rapidly increased due to the plant's response to a given stressor.

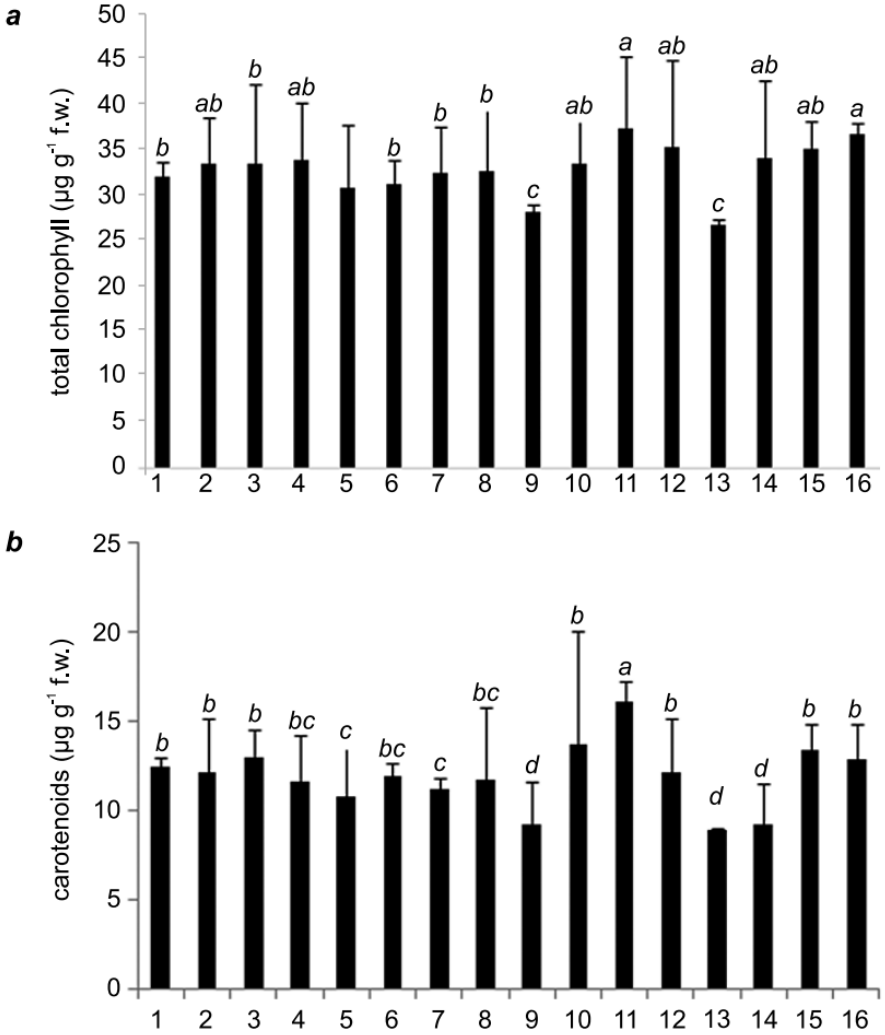

Fig. 3. Physiological parameters (*a* – total chlorophyll, *b* – carotenoids content) barley seedlings growing under stress induced by $Pb(NO_3)_2$ in the presence of nicotinamide.
Values denoted with the same letters within an experimental do not differ statistically ($p < 0,05$); no. 1-16 – see Table 1

According to Purnama et al. (2015) and Sędzik et al. (2015), a high Pb concentration in the substrate also has a significant influence on the content of photosynthetic (chlorophyll) and non-photosynthetic (carotenoids) pigments. Heavy metals lead to the direct inhibition of the activity of enzymes responsible for the chlorophyll *a* and *b* synthesis and to the malfunctioning of photosynthesis (Purnama et al. 2015). In the present study, increasing the $Pb(NO_3)_2$ content in the MS medium caused a decrease in the total chlorophyll and carotenoid concentrations in 10-day-old barley (Figure 3). Seedlings on the MS medium supplemented with 2.0 μg g^{-1} fm showed a 17% and 29% reduction in the total chlorophyll and carotenoids, respectively, compared to the control (32.24 μg g^{-1} fm and 12.52 μg g^{-1} fm, respectively).

However, the addition of nicotinamide to the MS medium supplemented with 1.0 and 2.0 mM $Pb(NO_3)_2$ had a significant effect on chlorophyll and carotenoids. Similar results were obtained by Azooz et al. (2013) in *Vicia faba* L., and by Sędzik et al. (2015) in experiments on various crop plant.

CONCLUSIONS

In conclusion, the present work has shown that lead exposition to barley seedling induces numerous metabolic disturbances, which result in a dose--dependent inhibition of seeds' germination, root and shoot length, fresh weight, as well as MDA, proline, CAT and total chlorophyll and carotenoids. In this study, the antioxidant properties and role of nicotinamide in lead stress were shown as the compound could inhibit the damaging effects of lead. The application of nicotinamide as an antioxidant increased the apical growth, as well as the development and biochemical parameters of *Hordeum vulgare* var. Eunova in *in vitro* culture. However, it was observed that nicotinamide in concentrations of 50 or 100 μM produced the best effect. The increase of tolerance to heavy metal stress by nicotinamide may be due to the enhanced antioxidant capacity, but may also be through the restoration of the hormone balance, which has to be verified in a future study.

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Examining Nicotinamide Application Methods in Alleviating Lead-Induced Stress in Spring Barley

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Abstract: Cereals are a staple food in many regions of the world and are essential for global food security. Lead is one of the most significant environmental stressors, impacting plants throughout their life cycle and causing substantial damage to plant growth and development. It disrupts intracellular processes, thereby reducing plant productivity. The aim of this study was to determine the effect of exogenously applied vitamin PP (100 µM) (nicotinamide) on the morphological, physiological, and biochemical parameters of spring barley var. Eunova under lead stress (1 mM $Pb(NO_3)_2$) and to determine the most effective method of applying this vitamin in a pot experiment. Vitamin PP was applied exogenously through three different methods: seed soaking, foliar application, and soil irrigation. The application of 1 mM $Pb(NO_3)_2$ resulted in decreased root (from 13.9% to 19.9%) and shoot length (from 16.2% to 24.8%) and increased catalase (CAT) activity from 45% to 106%, and peroxidase (POX) activity from 39% to 46% compared to the control. Lead stress led to an increase in proline (Pro) content from 30 to 63% and comparatively in malondialdehyde (MDA) content (rising from 61% to 79.4%), as well as elevated assimilatory pigment content (by 35%) in barley grown in the pot experiment. Exogenous vitamin PP significantly and positively influenced the improvement of the measured morphological, biochemical, and physiological parameters, reducing the toxicity of lead salts. It was shown that the most effective method of vitamin PP application was achieved through foliar spraying and irrigation.

Keywords: barley; lead stress; nicotinamide; morphological; biochemical; physiological parameters

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1. Introduction

Global agriculture faces numerous challenges, notably the need to increase food production by more than 60% to meet the needs of a growing population [1,2]. To meet this growing demand, agriculture will need millions of additional hectares of arable land [1]. However, since a significant proportion of arable land is already contaminated, utilizing such land will play a significant role in modern agriculture. Advances and innovations in agriculture have the potential to prevent a global shortage of arable land [3].

Reduced agricultural productivity stems from various environmental factors, with heavy metals playing a significant role [4–6]. Heavy metal contamination poses a serious problem due to their toxicity and persistence in the environment [7,8], affecting both developed and developing countries [9].

Lead (Pb) ranks as the second most toxic heavy metal in the environment [10]. It can enter the environment through various sources, stemming from both natural processes and human activities, such as mining and metallurgy, industrial emissions, fossil fuel combustion, pesticide use, improper waste disposal in landfills, the presence of lead-based paints, and the use of lead in water pipes or sewerage systems [11,12]. These activities contribute to the distribution of lead across different environmental compartments, like air, soil, water, and sediments. Plants readily absorb this element from the soil, accumulating it in various organs [11,13]. Exposure to lead inhibits plant growth, biomass, and development, and

has adverse effects on physiological and metabolic processes [14]. Subsequently, oxidative stress ensues in plants due to the generation of reactive oxygen species (ROS), such as hydroxyl radicals (OH^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and superoxide anion radicals (O_2^-). ROS overproduction disrupts cellular redox homeostasis, leading to structural damage in plants [15,16]. All ROS can cause damage to assimilatory pigments, cell membranes, carbohydrates, DNA, proteins, disruption of oxidative phosphorylation, and damage to the mitochondrial respiratory chain [11,16].

Plants have evolved their own cellular defense mechanisms to avoid, tolerate, detoxify, or eliminate heavy metals [17,18]. These mechanisms involve changes at the molecular, biochemical, and physiological levels [18,19]. They encompass chelation by metallothionein or phytochelatin, compartmentalization in cell walls and intracellular vesicles, lignification, inhibition of direct uptake of heavy metals from the soil, and the neutralization mechanism for ROS [17]. This latter mechanism, incorporating both nonenzymatic and enzymatic elements, converts ROS into less toxic compounds [15–17].

Despite the array of defense mechanisms possessed by plants, their resilience is often overwhelmed by excessive levels of heavy metals. To support plant tolerance or alleviate heavy metal-induced stress at the physiological, biochemical, and molecular levels within the cell, various exogenous substances have been tested in numerous scientific studies [4,19–21]. According to Feng et al. (2023), exogenous substances are defined as various compounds necessary for plant growth and development. These substances are introduced from external sources and act to increase plant tolerance. The primary functions of exogenous substances are associated with regulatory dimensions, specifically, enhancing the effectiveness of the antioxidant system, inducing the production of osmoregulatory compounds, improving the efficiency of the photochemical pathway, redirecting the accumulation and movement of heavy metals, modulating endogenous hormone levels, and controlling gene expression [22].

In recent years, numerous scientific studies have explored the use of exogenous substances to mitigate the toxicity of various abiotic stresses, including those induced by heavy metals. Effective alleviation of lead stress has been achieved through the application of substances such as brassinosteroids [23–26], auxins, cyto-kinins [27], salicylic acid (SA) [28,29] jasmonic acid (JA) [30], various organic chelates [31,32], glutathione (GSH) [33], and vitamins [34–36]. Various methods of introducing exogenous substances into plants exposed to heavy metals are documented in the literature. For example, these substances can be applied in hydroponic systems [31,35,37], on Petri dishes [36], in vitro culture on MS media [35,38], or in soil [29,33]. They can also be applied as foliar sprays [29,37,39] or seed imbibition treatments [39].

Niacinamide, also known as niacin or vitamin PP, stands as one of the major nonenzymatic antioxidants. This compound acts as a precursor to the coenzymes NADH and NADPH, crucial in numerous enzymatic oxidation–reduction reactions in plant cells. These coenzymes are vital for energy metabolism, photosynthesis, and cellular respiration [40,41]. Niacinamide plays a role in the biosynthesis of secondary metabolites [40,42], contributing to plant defense, signaling, and adaptation to environmental challenges [43]. Therefore, vitamin PP is crucial for plant growth, development, and adaptation to changing environmental conditions, highlighting its importance.

Barley, scientifically known as *Hordeum vulgare* L., stands as one of the oldest cultivated crops. It is primarily grown for human consumption, as an ingredient in animal feed, and for the production of alcoholic beverages. Due to its rapid growth, low climatic requirements, adaptability to various environments, and clear response to stress factors, it serves as a valuable subject for abiotic stress research [44,45].

The aim of the research was to assess the impact of exogenously applied vitamin PP (100 µM) on the morphological, physiological, and biochemical parameters of spring barley (*H. vulgare* L.) var. Eunova under lead stress (1 mM $Pb(NO_3)_2$) and to establish the most effective method of applying this vitamin in the pot experiment.

2. Materials and Methods

2.1. Experiment Site and Conditions

The 2-year pot experiment took place during the spring in the growth chamber and laboratory of Microbiology and Environmental Biochemistry, West Pomeranian University of Technology in Szczecin (latitude 53°26′17″ N, longitude 14°32′32″ E).

The soil used for the study was sourced from the arable–humus layer (Ap, 0–30 cm) in Ostoi, near Szczecin. This soil underwent sieving through a 2 mm mesh sieve and was divided into eight portions. Four portions were subjected to a solution of 1 mM $Pb(NO_3)_2$ (207.0 mg Pb^{+2}), while the remaining portions were treated with water to attain 60% of the maximum water-holding capacity. The prepared soil was then placed into 3.50 kg pots.

The material for the study consisted of seeds of spring barley var. Eunova, acquired a class (C/1) certified seed from a specialized shop. Before sowing, the seeds underwent three rinses of 20 min each in sterile distilled water. Subsequently, they were immersed in a 7% sodium hypochlorite solution for 10 min, followed by rinsing in sterile distilled water for 15 min. After this initial sterilization process, some seeds were immersed in a solution of 100 µM Vit PP in the form of nicotinamide, while the remaining seeds were soaked in water for 24 h. An equal number of seeds (10 barley seeds per pot) were sown in each pot at a depth of 3.0 cm.

The pots received natural watering every 10 days (100 cm^3 of distilled water) until the first leaves appeared. Thereafter, the barley var. Eunova plants were watered every 10 days (100 cm^3 of distilled water or water supplemented with 100 µM Vit PP). Additionally, some plants were sprayed with a solution containing 100 µM Vit PP. Each plant in the pot was thoroughly sprayed with the solution until it dripped from the leaves into the pot, with 10 cm^3 of solution per plant, amounting to a total of 100 cm^3 per pot. All solutions used for spraying and watering included Tween 20 as a dispersing agent.

The experiment consisted of 8 combinations carried out in 3 replicates and detailed information on the compounds used in this study is given below: (1) control, (2) 100 µM Vit PP soaking seeds, (3) 100 µM Vit PP foliar application, (4) 100 µM Vit PP soil application, (5) 1 mM $Pb(NO_3)_2$, (6) 1 mM $Pb(NO_3)_2$ + 100 µM Vit PP soaking seeds, (7) 1 mM $Pb(NO_3)_2$ + 100 µM Vit PP foliar application, (8) 1 mM $Pb(NO_3)_2$ + 100 µM Vit PP soil application.

During the growing season, the growth and development of the plants were observed. biochemical parameters (catalase and peroxidase activity, malondialdehyde and proline content) and physiological parameters (total chlorophyll and carotenoid content) were measured at four stages related to the physiological development of the plants: tillering, stem elongation, heading and flowering. In addition, morphological measurements of the plants (root length, stem length, spike length, fresh root weight, fresh stem weight, fresh spike weight) were made after the flowering phase (Figure 1). The experiment was conducted in a complete randomization system. All measurements were performed in three replicates.

2.2. Determination of the Morphological Parameters

After the plants had flowered, they were harvested. The spike, stem, and roots of all plants in each replicate were measured in centimeters using a ruler, and the measurements were recorded. The spike, roots, and stem were separated to measure their mass, and the roots were thoroughly cleaned of soil before weighing them on a digital scale.

2.3. Determination of Biochemical and Physiological Parameters

To assess the activity of antioxidant enzymes and determine the levels of Pro and MDA, fresh plant tissue from fully developed apical leaves was collected and homogenized with an appropriate chilled extraction solution. The extraction solutions used were 0.0067 M phosphate buffer at pH 7.0 for CAT, 0.05 M acetate buffer at pH 5.6 for POX, 3% sulfosalicylic acid for Pro, and 0.1% trichloroacetic acid for MDA. The resulting homogenates were then centrifuged at 15,000× *g* at 40 °C for 15–25 min. The supernatants obtained were used for each assay.

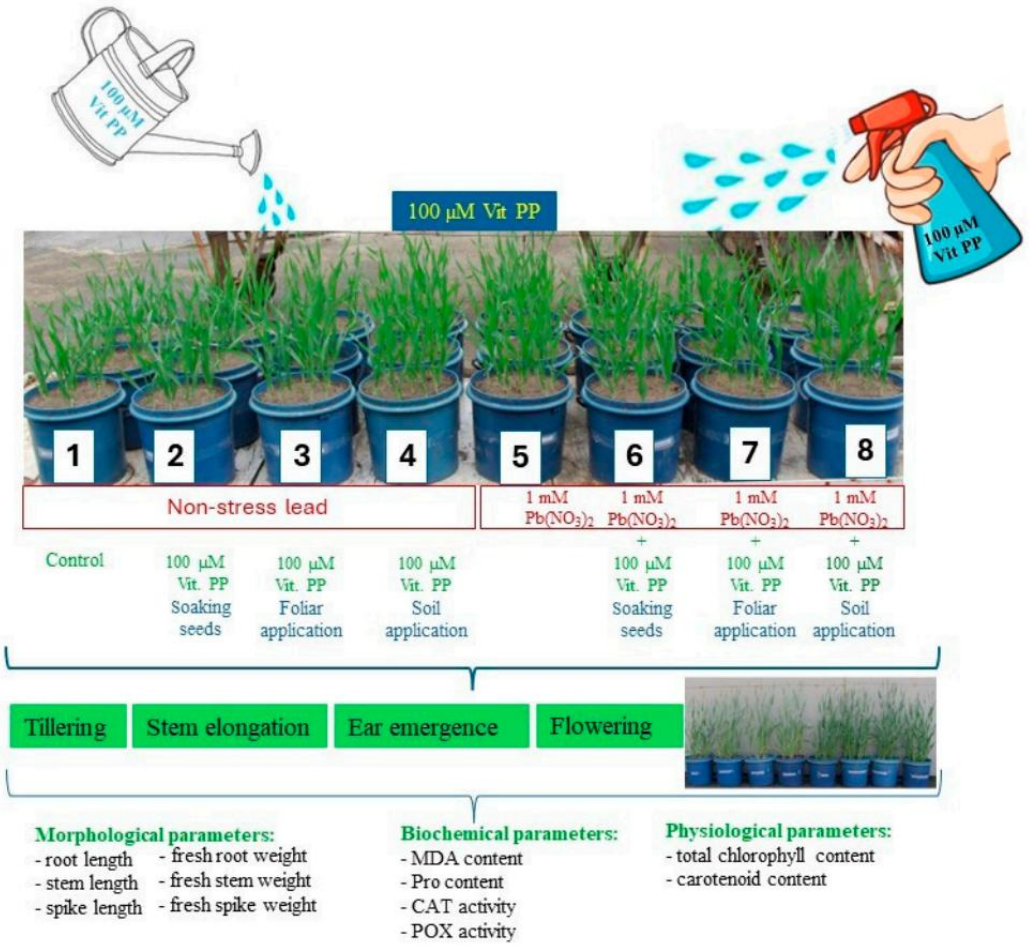

Figure 1. Schematic of the pot experiment.

Catalase activity (CAT [EC 1.11.1.6]) was determined using a spectrophotometer, (Merck Nova 400, Darmstadt, Germany) following Lück's method [46]. The reaction mixture consisted of 1/15 M phosphate buffer (pH 7.0), 1.25×10^{-2} M H_2O_2, and the centrifuged enzyme extract. The assay involved measuring the decrease in ultraviolet light absorption over 60 s as H_2O_2 decomposed by CAT at a wavelength of $\lambda = 240$ nm. Enzyme activity was expressed as µM $H_2O_2 \cdot g^{-1}$ FW of plant tissue$\cdot min^{-1}$.

Peroxidase activity (POX [EC 1.11.1.7]) activity was measured following the method of Chance and Maehly [47] using a spectrophotometer, (Merck Nova 400, Darmstadt, Germany). The method involved the colorimetric determination of purpurogallin formation during the oxidation of pyrogallol (0.02 M) in the presence of H_2O_2 (0.06 M) at a wavelength of $\lambda = 430$ nm over 4 min. Peroxidase activity was expressed in µM purpurogallol$\cdot g^{-1}$ FW of plant tissue$\cdot min^{-1}$.

The content of free proline was determined using the ninhydrin reaction per Bates method [48]. The reaction mixture consisted of extracted plant material in 3% sulfosalicylic acid, ice-cold acetic acid, and acidic ninhydrin. After boiling the mixture in a water bath at 100 °C for 60 min, the reaction was stopped by cooling the samples on ice. The samples were then extracted with toluene, and the absorbance of the colored chromophore against toluene was measured on a spectrophotometer, (Merck Nova 400, Darmstadt, Germany)

at a wavelength of λ = 520 nm. The concentration of proline was read from the standard curve prepared for L-proline and expressed in $\mu mol \cdot g^{-1}$ FW of plant tissue.

Malondialdehyde levels were determined through reaction with thio-barbituric acid following Sudhakar et al. [49] method (2001). Homogenates containing 0.5% TBA dissolved in 20% TCA were boiled at 100 °C for 10 min and rapidly cooled on ice. After centrifugation (10,000× *g*, 10 min), the absorbance of the supernatant was measured using a Merck Nova 400 spectrophotometer at wavelengths of λ = 532 nm and λ = 600 nm. The concentration of MDA was calculated using the millimolar absorption coefficient ε = 155 mM^{-1} cm^{-1}. The result was adjusted by subtracting the absorbance of the sample at 600 nm, which accounts for nonspecific reaction products with TBA. Malondialdehyde content was expressed as nmol $MDA \cdot g^{-1}$ FW of plant tissue.

Assimilatory pigments (chlorophylls and carotenoids) were extracted from plant material using chilled 80% acetone. Chlorophyll content was determined by the method of Arnon et al. [50] as modified by Lichtenthaler and Wellburn [51], while carotenoid content was determined by the method of Hager and Meyer-Berthenrath [52]. To extract assimilatory pigments from leaves, green samples weighing approximately 0.05 g were ground in a mortar with 10 cm^3 of 80% acetone. The homogenates were then centrifuged at 1500 rpm for 10 min.

2.4. Statistical Analysis of Results

Statistical analyses were performed using Statistica 13 (TIBCO Software Inc., Krakow, Poland). The results were analyzed using descriptive statistics, including mean and standard deviation. Means were compared using Tukey's HSD test at a significance level of $p < 0.05$.

3. Results

3.1. Plant Growth and Biomass

The statistical analysis of barley's morphological parameters, conducted after the flowering phase, showed that in both the first and second seasons, as well as in the combined data from both seasons, there were generally no significant differences in the measured parameters among the tested combinations (Figures 2 and 3). However, significant differences were observed in root length and stem length between the tested combinations. Specifically, the addition of lead resulted in a 13.9% decrease in root length in the first season and a 19.9% decrease in the second season, with stem length decreasing by 24.8% and 16.2%, respectively, compared to the control. Moreover, the application of exogenous vitamin PP had a significant and positive impact on root and stem length, mitigating the toxicity of lead salts. The most effective application of vitamin PP was through foliar spraying, demonstrating its beneficial effects on plant growth and lead stress reduction.

3.2. Antioxidant Enzyme Activities, Malondialdehyde, and Proline Contents

It was observed that the activity of enzymes involved in antioxidant defense significantly increased ($p > 0.05$) in the presence of lead salts. CAT activity increased from 45% to 106% compared to the control, while POX activity showed a similar level of increase (from 39% to 46%) during the developmental stages of barley studied (across both years). The application of vitamin PP in various forms slightly increased the enzyme activity compared to the control, but these differences were described as insignificant. However, applying this vitamin to plants on lead-contaminated soil led to decreased enzyme levels compared to plants exposed to lead alone. The most significant decrease in enzyme activity was observed with foliar spraying and irrigation using vitamin PP, in both the first and second years of the study (Table 1).

Lead salts contributed to an increase in Pro content in both years of the experiment. However, significant differences were observed only at the heading and flowering stages of barley, with no notable differences in Pro content found at earlier stages of plant development. The application of vitamin PP resulted in a reduction of Pro in barley var. Eunova

plants are compared to plants growing solely on lead. The most significant effect was observed during spraying and irrigation (Table 2).

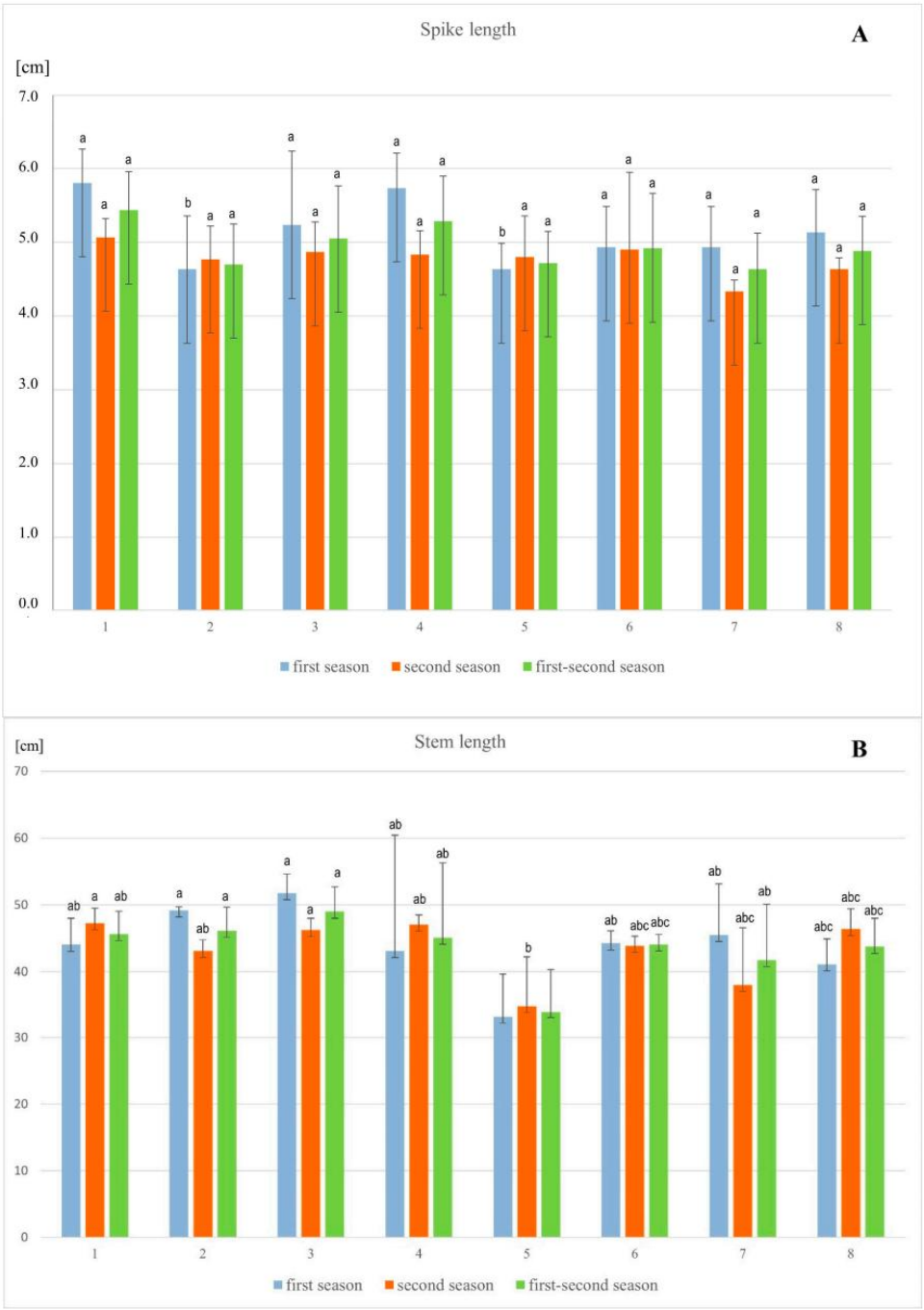

Figure 2. *Cont.*

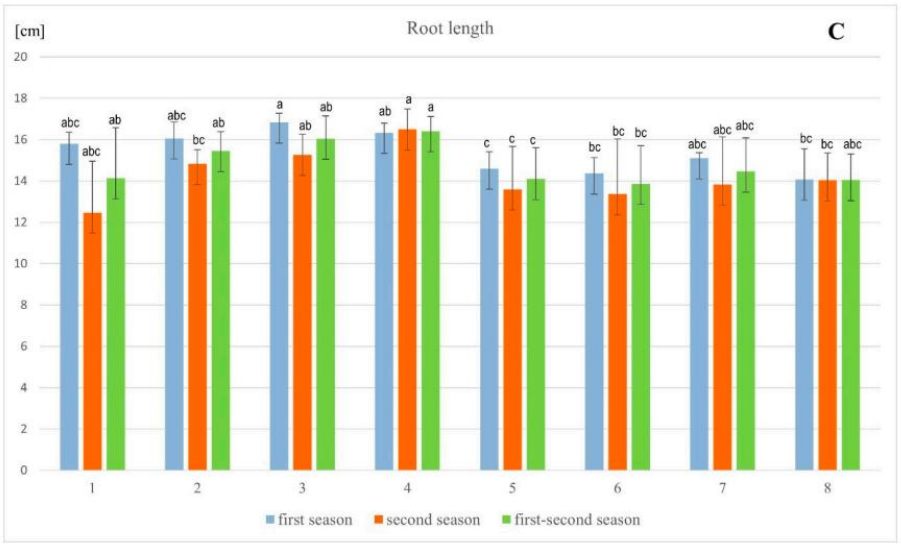

Figure 2. Influence of different methods of vitamin PP application on the length (**A**–**C**) of spring barley var. Eunova growing in soil with 1 mM $Pb(NO_3)_2$; (**A**)–spike, (**B**)–stem, (**C**)–root; (1) control, (2) 100 µM Vit PP soaking seeds, (3) 100 µM Vit PP foliar application, (4) 100 µM Vit PP soil application, (5) 1 mM $Pb(NO_3)_2$, (6) 1 mM $Pb(NO_3)_2$ + 100 µM Vit PP soaking seeds, (7) 1 mM $Pb(NO_3)_2$ + 100 µM Vit PP foliar application, (8) 1 mM $Pb(NO_3)_2$ + 100 µM Vit PP soil application; a–c—homogeneous groups.

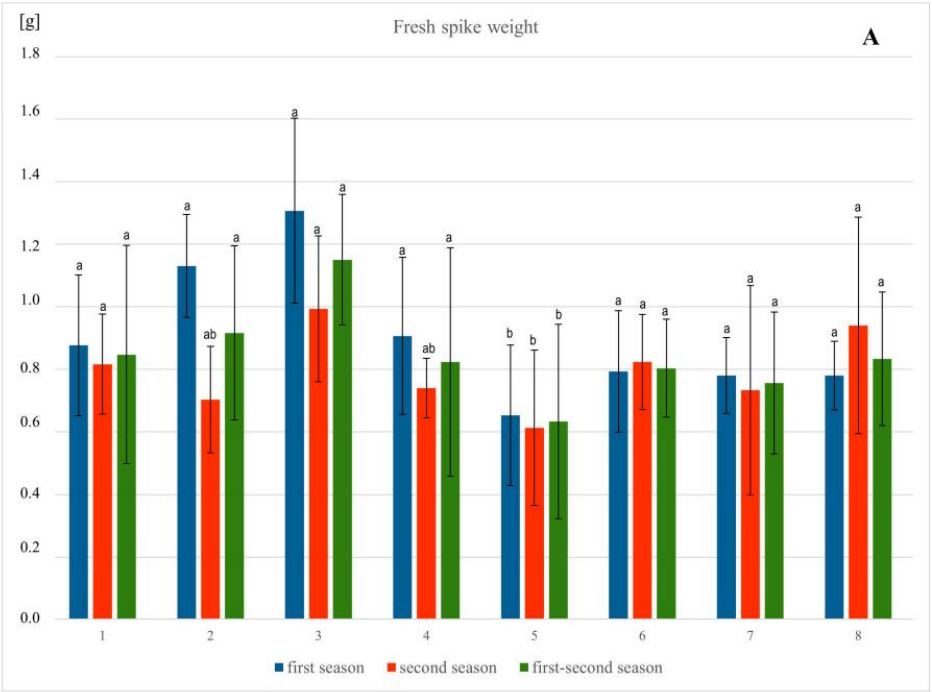

Figure 3. *Cont.*

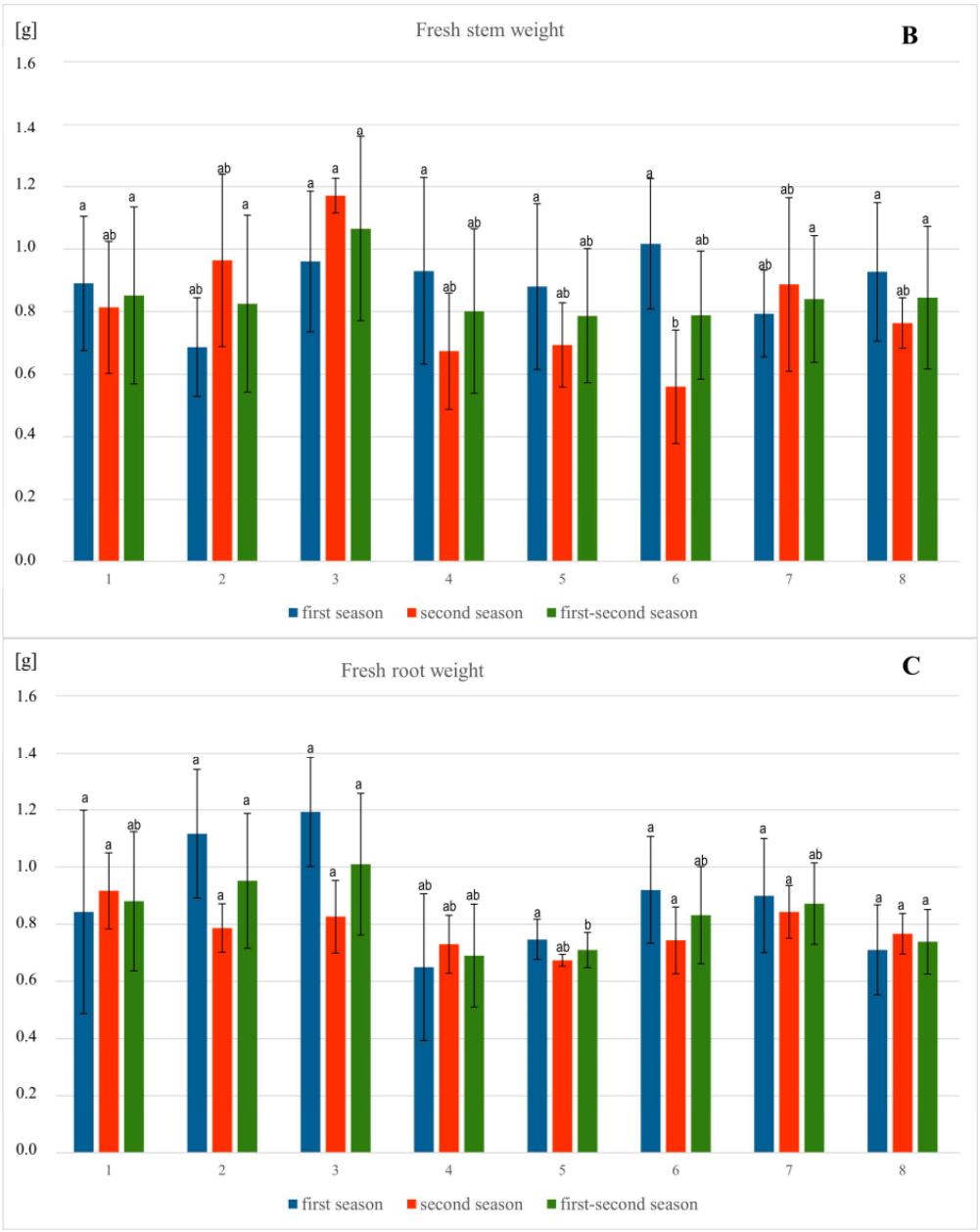

Figure 3. Influence of different methods of vitamin PP application on the fresh weight (**A**–**C**) of spring barley var. Eunova growing in soil with 1 mM $Pb(NO_3)_2$; **A**–spike, **B**–stem, **C**–root; (1) control, (2) 100 µM Vit PP soaking seeds, (3) 100 µM Vit PP foliar application, (4) 100 µM Vit PP soil application, (5) 1 mM $Pb(NO_3)_2$, (6) 1 mM $Pb(NO_3)_2$ + 100 µM Vit PP soaking seeds, (7) 1 mM $Pb(NO_3)_2$ + 100 µM Vit PP foliar application, (8) 1 mM $Pb(NO_3)_2$ + 100 µM Vit PP soil application; a–b—homogeneous groups.

Lead stress contributed significantly ($p > 0.05$) to an increase in MDA content compared to the control (rising from 61% to 79.4%) during the developmental stages studied in barley var. Eunova (across both years). The exogenous application of vitamin PP, particularly through watering and in most cases, spraying, significantly ($p > 0.05$) reduced the MDA content compared to plants grown with lead alone (Table 2).

3.3. Total Chlorophyll and Carotenoid Contents

The lead salts used in the experiment significantly ($p > 0.05$) reduced the content of assimilatory pigments, including total chlorophyll and carotenoids (Table 3). As the plants grew, there was a gradual decrease in total chlorophyll content (ranging from 20.3% to 35.3% lower than the control), while carotenoids decreased by 22.4% to 28.7% (across both years). Vitamin PP, applied in all forms, increased the content of both total chlorophyll and carotenoids compared to plants growing with lead alone, but the increase was significant only when applied through irrigation.

Table 1. Influence of different methods of application of vitamin PP on the enzyme activity of barley growing in soil with lead.

Catalase [μmol·H_2O_2·g^{-1} FW·min^{-1}] (% Control)												
Combination	**First Season**				**Second Season**				**Synthesis First—Second Season**			
	Tillering	**Stem Elongation**	**Ear Emergence**	**Flowering**	**Tillering**	**Stem Elongation**	**Ear Emergence**	**Flowering**	**Tillering**	**Stem Elongation**	**Ear Emergence**	**Flowering**
1	104.8 ± 5.49 ^{c} (100)	100.4 ± 5.30 ^{d} (100)	84.7 ± 2.74 ^{c} (100)	60.7 ± 0.12 ^{d} (100)	115.4 ± 10.26 ^{b} (100)	110.6 ± 8.83 ^{d} (100)	63.36 ± 5.28 ^{c} (100)	48.9 ± 11.45 ^{c} (100)	110.1 ± 8.71 ^{c} (100)	105.5 ± 8.59 ^{d} (100)	74.1 ± 12.30 ^{c} (100)	54.8 ± 9.69 ^{c} (100)
2	119.3 ± 5.14 ^{bc} (113.9)	107.9 ± 7.53 ^{cd} (107.5)	92.6 ± 4.54 ^{c} (109.3)	67.7 ± 1.87 ^{c} (111.5)	121.5 ± 3.12 ^{b} (105.30)	114.4 ± 1.77 ^{cd} (103.34)	68.9 ± 1.69 ^{c} (108.9)	52.5 ± 4.17 ^{c} (107.3)	120.4 ± 3.98 ^{c} (109.3)	111.2 ± 6.02 ^{d} (105.4)	80.8 ± 13.27 ^{c} (109.0)	60.1 ± 8.80 ^{c} (109.7)
3	108.1 ± 9.93 ^{c} (103.2)	102.4 ± 1.84 ^{d} (102.02)	85.0 ± 3.93 ^{c} (100.35)	60.7 ± 1.97 ^{d} (100.1)	113.2 ± 4.87 ^{b} (98.1)	110.2 ± 5.67 ^{d} (99.6)	61.9 ± 2.76 ^{c} (97.7)	51.7 ± 5.39 ^{c} (105.56)	110.6 ± 7.53 ^{c} (100.5)	106.3 ± 5.70 ^{d} (100.7)	73.5 ± 13.04 ^{c} (99.2)	56.1 ± 6.15 ^{c} (102.4)
4	109.8 ± 4.93 ^{c} (104.8)	104.9 ± 4.27 ^{d} (104.6)	88.8 ± 6.66 ^{c} (104.8)	66.4 ± 1.24 ^{cd} (109.4)	119.2 ± 7.76 ^{b} (103.3)	121.2 ± 4.43 ^{cd} (109.5)	67.7 ± 5.69 ^{c} (106.9)	51.1 ± 1.88 ^{c} (104.4)	114.5 ± 7.77 ^{c} (103.9)	113.1 ± 9.69 ^{d} (107.2)	78.3 ± 12.30 ^{c} (105.7)	58.7 ± 8.52 ^{c} (107.1)
5	150.2 ± 20.80 ^{a} (143.3)	185.6 ± 2.34 ^{a} (184.8)	178.4 ± 8.21 ^{a} (210.5)	122.5 ± 2.38 ^{a} (201.8)	170.63 ± 3.28 ^{a} (147.91	167.2 ± 2.04 ^{a} (151.1)	121.6 ± 3.51 ^{a} (191.9)	102.3 ± 2.43 ^{a} (209.1)	160.4 ± 17.39 ^{a} (145.7)	176.4 ± 10.14 ^{a} (167.2)	150.0 ± 31.59 ^{a} (202.4)	112.4 ± 11.25 ^{a} (205.1)
6	143.6 ± 1.79 ^{ab} (137.0)	132.9 ± 4.16 ^{b} (132.4)	143.9 ± 6.39 ^{b} (169.9)	90.6 ± 3.68 ^{b} (149.3)	159.0 ± 4.95 ^{ab} (137.8)	143.7 ± 3.73 ^{b} (129.9)	91.4 ± 8.72 ^{b} (144.2)	77.3 ± 7.44 ^{b} (157.9)	151.3 ± 9.08 ^{ab} (137.4)	138.3 ± 6.87 ^{b} (131.1)	117.7 ± 29.61 ^{b} (158.8)	83.9 ± 8.98 ^{b} (153.1)
7	126.9 ± 2.17 ^{abc} (121.13)	121.0 ± 4.48 ^{bc} (120.5)	139.1 ± 5.75 ^{b} (164.1)	91.2 ± 1.81 ^{b} (150.2)	155.5 ± 6.56 ^{ab} (134.8)	124.4 ± 8.78 ^{c} (112.4)	83.3 ± 3.13 ^{b} (131.5)	76.1 ± 5.3 ^{b}(155.6)	141.2 ± 16.26 ^{b} (128.2)	122.7 ± 3.41 ^{c} (116.3)	111.2 ± 30.82 ^{b} (150.1)	83.6 ± 8.96 ^{b} (152.5)
8	122.5 ± 5.11 ^{bc} (116.9)	126.0 ± 6.23 ^{b} (125.5)	135.4 ± 5.52 ^{b} (159.7)	87.9 ± 4.01 ^{b} (144.8)	157.7 ± 4.23 ^{ab} (136.7)	138.4 ± 11.02 ^{b} (125.1)	94.5 ± 4.79 ^{b} (149.1)	74.1 ± 2.09 ^{b} (151.4)	140.1 ± 19.71 ^{b} (127.2)	132.2 ± 7.86 ^{b} (125.3)	114.9 ± 22.86 ^{b} (155.1)	80.9 ± 8.07 $^{b-}$ (147.6)
Peroxidase [μM Purpurogallin·g^{-1} FW·min^{-1}] (% Control)												
Combination	**First Season**				**Second Season**				**SynthesisFirst—Second Season**			
	Tillering	**Stem Elongation**	**Ear Emergence**	**Flowering**	**Tillering**	**Stem Elongation**	**Ear Emergence**	**Flowering**	**Tillering**	**Stem Elongation**	**Ear Emergence**	**Flowering**
1	3.7 ± 0.84 ^{b} (100)	6.0 ± 0.51 ^{c} (100)	8.0 ± 0.32 ^{b} (100)	7.9 ± 1.08 ^{c} (100)	3.6 ± 0.22 ^{c} (100)	6.4 ± 0.31 ^{b} (100)	7.4 ± 0.12 ^{c} (100)	8.1 ± 1.32 ^{b} (100)	3.7 ± 0.53 ^{d} (100)	6.2 ± 0.43 ^{b} (100)	7.7 ± 0.39 ^{d} (100)	8.0 ± 0.84 ^{d} (100)
2	4.1 ± 0.46 ^{ab} (110.4)	6.2 ± 1.36 ^{bc} (103.7)	8.4 ± 0.45 ^{b} (105.6)	8.3 ± 0.61 ^{bc} (105.1)	3.9 ± 0.22 ^{bc} (107.9)	6.6 ± 1.24 ^{b} (102.8)	7.6 ± 0.22 ^{bc} (103.5)	8.3 ± 0.69 ^{b} (102.6)	4.0 ± 0.32 ^{cd} (108.1)	6.4 ± 0.84 ^{b} (103.2)	8.0 ± 0.53 ^{cd} (103.9)	8.3 ± 0.58 ^{cd} (103.7)
3	4.1 ± 0.15 ^{ab} (109.6)	6.2 ± 0.12 ^{bc} (102.6)	7.9 ± 0.71 ^{b} (99.4)	8.1 ± 0.68 ^{bc} (101.4)	3.91 ± 0.19 ^{bc} (107.7)	6.3 ± 0.74 ^{b} (98.2)	7.5 ± 0.72 ^{bc} (102.1)	8.1 ± 0.23 ^{b} (100.3)	4.0 ± 0.15 ^{cd} (108.1)	6.2 ± 0.48 ^{b} (100.1)	7.7 ± 0.67 ^{d} (100.1)	8.1 ± 0.45 ^{d} (101.2)
4	4.1 ± 0.45 ^{ab} (108.9)	6.1 ± 0.53 ^{bc} (102.3)	7.9 ± 0.45 ^{b} (99.4)	8.0 ± 0.38 ^{c} (101.6)	3.8 ± 0.13 ^{bc} (105.2)	6.5 ± 0.21 ^{b} (101.2)	7.5 ± 0.71 ^{bc} (101.1)	8.2 ± 0.44 ^{b} (100.8)	3.9 ± 0.33 ^{cd} (105.4)	6.3 ± 0.40 ^{b} (101.6)	7.7 ± 0.59 ^{d} (100.1)	8.1 ± 0.37 ^{d} (101.2)
5	5.4 ± 0.49 ^{a} (144.8)	8.4 ± 0.54 ^{a} (140.4)	11.1 ± 1.03 ^{a} (138.7)	10.9 ± 0.96 ^{a} (137.9)	5.3 ± 0.19 ^{a} (146.38)	8.8 ± 0.85 ^{a} (136.9)	10.3 ± 0.82 ^{a} (139.6)	12.1 ± 1.50 ^{a} (149.7)	5.4 ± 0.34 ^{a} (145.9)	8.6 ± 0.66 ^{a} (138.7)	10.7 ± 0.95 ^{a} (138.9)	11.5 ± 1.16 ^{a} (143.7)
6	5.1 ± 0.49 ^{ab} (135.3)	7.8 ± 0.29 ^{a} (130.8)	9.6 ± 0.33 ^{ab} (120.1)	9.9 ± 0.89 ^{ab} (125.8)	4.6 ± 0.52 ^{ab} (126.1)	8.1 ± 0.39 ^{ab} (125.8)	9.1 ± 0.32 ^{ab} (123.1)	9.9 ± 0.72 ^{ab} (122.0)	4.8 ± 0.52 ^{ab} (129.7)	7.9 ± 0.32 ^{a} (127.4)	9.3 ± 0.39 ^{b} (120.8)	9.9 ± 0.68 ^{b} (123.7)

Table 1. *Cont.*

Combination	Peroxidase [μM Purpurogallin·g^{-1} FW·min^{-1}] (% Control)											
	First Season				Second Season				SynthesisFirst—Second Season			
	Tillering	Stem Elongation	Ear Emergence	Flowering	Tillering	Stem Elongation	Ear Emergence	Flowering	Tillering	Stem Elongation	Ear Emergence	Flowering
7	4.5 ± 0.39 ^{ab} (120.4)	7.3 ± 0.99 ^{abc} (121.8)	9.5 ± 1.24 ^{ab} (119.54)	9.7 ± 0.82 ^{abc} (122.1)	4.5 ± 0.43 ^{b} (123.2)	7.9 ± 0.74 ^{ab} (123.5)	8.8 ± 0.88 ^{abc} (118.8)	9.7 ± 0.65 ^{b} (119.7)	4.5 ± 0.28 ^{bc} (121.6)	7.6 ± 0.85 ^{a} (122.5)	9.2 ± 1.05 ^{bc} (119.5)	9.7 ± 0.66 ^{bc} (121.2)
8	4.7 ± 0.58 ^{ab} (126.6)	7.58 ± 0.36 ^{ab} (126.3)	9.1 ± 0.54 ^{ab} (114.0)	9.57 ± 1.15 ^{abc} (120.5)	4.3 ± 0.39 ^{bc} (119.9)	7.8 ± 0.21 ^{ab} (122.7)	8.7 ± 0.25 ^{bc} (117.5)	9.4 ± 0.42 ^{b} (115.3)	4.5 ± 0.49 ^{bc} (121.6)	7.7 ± 0.29 ^{a} (124..2)	8.9 ± 0.44 ^{bcd} (115.6)	9.5 ± 0.78 ^{bcd} (118.7)

(1) Control, (2) 100 μM Vit PP soaking seeds, (3) 100 μM Vit PP foliar application, (4) 100 μM Vit PP soil application, (5) 1 mM $Pb(NO_3)_2$, (6) 1 mM $Pb(NO_3)_2$ + 100 μM Vit PP soaking seeds, (7) 1 mM $Pb(NO_3)_2$ + 100 μM Vit PP foliar application, (8) 1 mM $Pb(NO_3)_2$ + 100 μM Vit PP soil application; a–d—homogeneous groups.

Table 2. Influence of different methods of application of vitamin PP on the Pro and MDA content of barley growing in soil with lead.

Combination	Proline [μmol·g^{-1}·FW] (% Control)											
	First Season				Second Season				Synthesis First—Second Season			
	Tillering	Stem Elongation	Ear emergence	Flowering	Tillering	Stem Elongation	Ear Emergence	Flowering	Tillering	Stem Elongation	Ear Emergence	Flowering
1	2.5 ± 0.20 ^{a} (100)	2.6 ± 0.79 ^{a} (100)	3.4 ± 1.44 ^{d} (100)	3.4 ± 0.42 ^{b} (100)	2.1 ± 0.68 ^{a} (100)	2.3 ± 0.27 ^{a} (100)	2.9 ± 0.13 ^{d} (100)	2.6 ± 0.21 ^{bc} (100)	2.3 ± 0.49 ^{b} (100)	2.5 ± 0.57 ^{cd} (100)	3.2 ± 0.95 ^{c} (100)	3.0 ± 0.51 ^{c} (100)
2	2.5 ± 0.11 ^{a} (98.7)	2.7 ± 0.49 ^{a} (102.7)	3.7 ± 0.18 ^{cd} (107.7)	3.6 ± 0.38 ^{b} (106.3)	2.1 ± 0.21 ^{a} (98.7)	2.5 ± 0.54 ^{a} (107.2)	3.1 ± 0.37 ^{bcd} (105.0)	2.8 ± 0.16 ^{b} (105.5)	2.3 ± 0.25 ^{b} (99.1)	2.6 ± 0.49 ^{bcd} (104.8)	3.4 ± 0.39 ^{bc} (106.3)	3.2 ± 0.52 ^{c} (105.6)
3	2.4 ± 0.64 ^{a} (96.93)	2.6 ± 0.11 ^{a} (98.0)	3.6 ± 1.13 ^{cd} (104.5)	3.5 ± 0.36 ^{b} (102.9)	2.0 ± 0.11 ^{a} (94.4)	2.3 ± 0.47 ^{a} (101.4)	3.0 ± 0.12 ^{cd} (102.4)	2.7 ± 0.43 ^{b} (102.9)	2.2 ± 0.47 ^{b} (96.1)	2.5 ± 0.34 ^{d} (99.6)	3.3 ± 0.78 ^{bc} (103.4)	3.1 ± 0.55 ^{c} (102.6)
4	2.5 ± 0.24 ^{a} (99.6)	2.6 ± 0.16 ^{a} (99.9)	3.7 ± 0.32 ^{cd} (107.0)	3.4 ± 0.25 ^{b} (100.6)	2.1 ± 0.17 ^{a} (96.9)	2.3 ± 0.18 ^{a} (101.5)	2.9 ± 0.15 ^{d} (99.6)	2.7 ± 0.22 ^{b} (102.0)	2.3 ± 0.30 ^{b} (98.7)	2.5 ± 0.23 ^{cd} (100.8)	3.3 ± 0.45 ^{bc} (103.4)	3.0 ± 0.45 ^{c} (100.7)
5	3.2 ± 0.89 ^{a} (130.3)	3.9 ± 0.37 ^{a} (147.9)	5.1 ± 0.33 ^{a} (149.5)	5.5 ± 0.59 ^{a} (163.2)	2.8 ± 0.15 ^{a} (135.5)	3.3 ± 0.35 ^{a} (143.4)	4.2 ± 0.38 ^{a} (142.6)	3.8 ± 0.38 ^{a} (145.5)	3.1 ± 0.60 ^{a} (133.0)	3.6 ± 0.48 ^{a} (146.1)	4.6 ± 0.60 ^{a} (146.2)	4.7 ± 0.96 ^{a} (154.8)
6	2.9 ± 0.52 ^{a} (119.9)	3.7 ± 0.88 ^{a} (140.4)	4.7 ± 0.14 ^{b} (138.0)	4.9 ± 0.76 ^{ab} (145.4)	2.8 ± 0.42 ^{a} (130.6)	3.1 ± 0.31 ^{a} (133.9)	3.8 ± 0.29 ^{ab} (130.2)	3.5 ± 0.29 ^{ab} (130.2)	2.9 ± 0.44 ^{ab} (125.2)	3.4 ± 0.69 ^{ab} (137.6)	4.3 ± 0.50 ^{ab} (134.3)	4.2 ± 0.96 ^{ab} (139.2)
7	2.7 ± 0.15 ^{a} (107.1)	3.8 ± 0.36 ^{a} (142.2)	4.4 ± 0.27 ^{b} (127.7)	4.8 ± 0.33 ^{ab} (141.1)	2.7 ± 0.21 ^{a} (129.2)	2.9 ± 0.38 ^{a} (129.5)	3.7 ± 0.21 ^{ab} (126.0)	3.2 ± 0.46 ^{ab} (120.1)	2.7 ± 0.17 ^{ab} (117.8)	3.4 ± 0.55 ^{ab} (136.4)	4.1 ± 0.43 ^{abc} (126.7)	3.9 ± 0.96 ^{b} (131.5)
8	2.8 ± 0.14 ^{a} (113.6)	3.7 ± 0.25 ^{a} (138.5)	4.6 ± 0.79 ^{b} (133.5)	4.7 ± 0.18 ^{ab} (140.3)	2.7 ± 0.22 ^{a} (127.1)	2.9 ± 0.52 ^{a} (128.4)	3.6 ± 0.32 ^{abc} (124.0)	3.0 ± 0.34 ^{ab} (130.8)	2.8 ± 0.18 ^{ab} (120.4)	3.3 ± 0.54 ^{abc} (134.0)	4.1 ± 0.74 ^{abc} (128.9)	3.9 ± 0.96 ^{b} (129.2)

Table 2. *Cont.*

Combination	MDA [μmol·g^{-1} FW] (% Control)											
	First Season				Second Season				Synthesis First—Second Season			
	Tillering	Stem Elongation	Ear Emergence	Flowering	Tillering	Stem Elongation	Ear Emergence	Flowering	Tillering	Stem Elongation	Ear Emergence	Flowering
1	18.3 ± 1.11 ^{b} (100)	25.3 ± 1.54 ^{c} (100)	28.2 ± 3.68 ^{b} (100)	30.1 ± 2.52 ^{c} (100)	11.1 ± 1.29 ^{c} (100.0)	24.9 ± 1.79 ^{c} (100)	33.3 ± 1.07 ^{b} (100)	36.5 ± 2.11 ^{c} (100)	14.7 ± 4.08 ^{d} (100)	25.1 ± 1.51 ^{c} (100)	30.8 ± 3.71 ^{b} (100)	33.3 ± 4.07 ^{d} (100)
2	19.3 ± 1.15 ^{b} (105.2)	26.2 ± 0.25 ^{c} (103.4)	29.2 ± 0.69 ^{b} (103.6)	32.1 ± 2.51 ^{c} (106.5)	11.9 ± 1.58 ^{bc} (106.6)	25.1 ± 2.23 ^{c} (96.5)	36.8 ± 3.79 ^{b} (110.5)	38.5 ± 4.09 ^{c} (105.4)	15.5 ± 4.12 ^{cd} (105.7)	25.6 ± 1.55 ^{c} (101.9)	33.0 ± 4.82 ^{b} (107.3)	35.3 ± 4.64 ^{d} (105.9)
3	18.3 ± 0.56 ^{b} (100.1)	25.2 ± 1.16 ^{c} (99.7)	28.9 ± 1.69 ^{b} (102.4)	31.1 ± 0.75 ^{c} (103.2)	11.8 ± 1.48 ^{bc} (105.8)	25.1 ± 2.62 ^{c} (96.5)	34.8 ± 1.89 ^{b} (104.4)	36.9 ± 4.23 ^{c} (101.2)	15.1 ± 3.73 ^{d} (102.3)	25.1 ± 1.82 ^{c} (100.1)	31.8 ± 3.61 ^{b} (103.5)	33.9 ± 4.21 ^{d} (102.1)
4	18.5 ± 1.25 ^{b} (100.9)	25.7 ± 0.92 ^{c} (101.6)	28.5 ± 3.14 ^{b} (101.2)	31.2 ± 1.55 ^{c} (103.8)	11.1 ± 1.44 ^{c} (99.4)	25.6 ± 0.63 ^{c} (98.5)	35.2 ± 6.48 ^{b} (105.6)	36.8 ± 3.37 ^{c} (100.8)	14.7 ± 4.24 ^{d} (100.3)	25.6 ± 0.71 ^{c} (101.9)	31.9 ± 5.83 ^{b} (103.6)	34.0 ± 3.83 ^{d} (102.2)
5	28.9 ± 2.9 ^{a} (158.3)	41.4 ± 1.21 ^{a} (163.4)	45.4 ± 5.4 ^{a} (160.1)	51.7 ± 5.12 ^{a} (171.6)	18.6 ± 3.01 ^{a} (167.1)	46.9 ± 4.07 ^{a} (180.8)	62.9 ± 5.39 ^{a} (188.9)	67.8 ± 2.19 ^{a} (185.8)	23.8 ± 6.27 ^{a} (161.9)	44.2 ± 4.05 ^{a} (175.6)	54.2 ± 10.78 ^{a} (176.0)	59.7 ± 9.52 ^{a} (179.4)
6	26.9 ± 3.11 ^{a} (146.8)	37.3 ± 2.32 ^{ab} (147.2)	42.6 ± 2.62 ^{a} (150.9)	46.6 ± 2.89 ^{ab} (154.7)	16.9 ± 1.85 ^{ab} (151.5)	41.6 ± 4.26 ^{ab} (160.4)	57.5 ± 2.21 ^{a} (172.6)	61.4 ± 5.88 ^{ab} (168.4)	21.9 ± 5.95 ^{ab} (148.6)	39.4 ± 3.88 ^{b} (156.9)	50.0 ± 8.47 ^{a} (162.6)	54.0 ± 9.13 ^{ab} (162.2)
7	26.1 ± 3.71 ^{a} (142.6)	33.9 ± 4.20 ^{b} (134.1)	40.3 ± 4.51 ^{ab} (142.9)	45.1 ± 2.52 ^{ab} (149.8)	16.4 ± 2.62 ^{ab} (147.3)	38.6 ± 3.34 ^{b} (148.8)	56.2 ± 3.95 ^{ab} (168.8)	53.7 ± 1.01 ^{b} (147.1)	21.3 ± 6.05 ^{ab} (144.4)	36.3 ± 4.25 ^{b} (144.3)	48.3 ± 9.52 ^{ab} (156.9)	49.4 ± 5.01 ^{bc} (148.3)
8	23.2 ± 2.19 ^{ab} (126.76)	32.7 ± 0.92 ^{b} (129.3)	40.6 ± 5.82 ^{ab} (143.9)	42.4 ± 4.85 ^{b} (141.0)	15.2 ± 1.23 ^{abc} (137.1)	38.3 ± 2.07 ^{b} (147.7)	56.5 ± 5.12 ^{ab} (169.6)	52.5 ± 2.21 ^{b} (143.9)	19.2 ± 4.64 ^{bc} (130.7)	35.5 ± 3.34 ^{b} (141.3)	48.6 ± 10.01 ^{ab} (157.9)	47.5 ± 6.47 ^{c} (142.6)

(1) Control, (2) 100 μM Vit PP soaking seeds, (3) 100 μM Vit PP foliar application, (4) 100 μM Vit PP soil application, (5) 1 mM $Pb(NO_3)_2$, (6) 1 mM $Pb(NO_3)_2$ + 100 μM Vit PP soaking seeds, (7) 1 mM $Pb(NO_3)_2$ + 100 μM Vit PP foliar application, (8) 1 mM $Pb(NO_3)_2$ + 100 μM Vit PP soil application; a–d—homogeneous groups.

Table 3. Influence of different methods of application of vitamin PP on the assimilation pigments content of barley growing in soil with lead.

Combination	Total Chlorophyll [μg·g^{-1} FW] (% Control)											
	First Season				Second Season				Synthesis First—Second Season			
	Tillering	Stem Elongation	Ear Emergence	Flowering	Tillering	Stem Elongation	Ear Emergence	Flowering	Tillering	Stem Elongation	Ear Emergence	Flowering
1	122.7 ± 5.42 ^{ab} (100)	119.5 ± 12.69 ^{ab} (100)	130.4 ± 15.65 ^{ab} (100)	107.2 ± 3.36 ^{a} (100)	120.4 ± 2.46 ^{ab} (100)	108.8 ± 13.95 ^{abc} (100)	130.2 ± 6.64 ^{a} (100)	104.9 ± 10.84 ^{ab} (100)	121.6 ± 3.95 ^{ab} (100)	114.2 ± 10.73 ab (100)	130.3 ± 10.75 ^{a} (100)	106.1 ± 7.28 ^{a} (100)
2	131.6 ± 4.67 ^{a} (107.3)	125.4 ± 2.73 ^{ab} (104.9)	132.8 ± 3.54 ^{ab} (101.9)	111.8 ± 2.93 ^{a} (104.3)	123.2 ±14.96 ^{ab} (102.3)	112.5 ± 7.38 ^{ab} (103.4)	133.5 ± 4.06 ^{a} (102.5)	106.7 ± 1.66 ^{ab} (101.6)	127.4 ± 10.93 ^{a} (104.10	118.9 ± 8.64 ^{a} (104.1)	133.1 ± 3.43 ^{a} (102.1)	109.2 ± 3.52 ^{a} (102.9)
3	133.8 ± 3.45 ^{a} (109.1)	125.9 ± 23.28 ^{ab} (105.3)	135.8 ± 5.84 ^{ab} (104.2)	108.6 ± 9.8 ^{a} (101.3)	128.8 ± 1.69 ^{a} (106.9)	120.7 ± 9.31 ^{a} (110.9)	146.9 ± 17.28 ^{a} (112.9)	112.1 ± 16.02 ^{ab} (106.8)	131.3 ± 8.17 ^{a} (107.9)	123.3 ± 16.11 ^{a} (107.9	141.4 ± 13.04 ^{a} (108.5)	110.3 ± 12.05 ^{a} (103.9)
4	135.3 ± 3.78 ^{a} (110.3)	129.7 ± 5.26 ^{a} (108.5)	140.8 ± 5.31 ^{a} (108.0)	115.8 ± 2.55 ^{a} (107.9	130.0 ± 6.83 ^{a} (107.9)	120.6 ± 1.44 ^{a} (110.9)	143.1 ± 7.46 ^{a} (109.9)	115.8 ± 5.72 ^{a} (110.3)	132.7 ± 5.72 ^{a} (109.1)	125.2 ± 6.03 ^{a} (109.6)	141.9 ± 5.92 ^{a} (108.9)	115.8 ± 3.96 ^{a} (109.1)

Table 3. *Cont.*

Total Chlorophyll [μg·g^{-1} FW] (% Control)												
Combination	**First Season**				**Second Season**				**Synthesis First—Second Season**			
	Tillering	**Stem Elongation**	**Ear Emergence**	**Flowering**	**Tillering**	**Stem Elongation**	**Ear Emergence**	**Flowering**	**Tillering**	**Stem Elongation**	**Ear Emergence**	**Flowering**
6	104.9 ± 2.77 ^{c} (85.5)	97.6 ± 10.76 ^{bc} (81.7)	104.6 ± 13.34 ^{bc} (80.27)	72.1 ± 1.24 ^{c} (67.2)	102.1 ± 5.51 ^{bc} (84.7)	85.5 ± 1.78 ^{d} (78.6)	91.4 ± 6.83 ^{b} (70.2)	82.9 ± 5.63 ^{bc} (78.9)	103.5 ± 4.19 ^{c} (85.1)	91.6 ± 9.56 ^{cd} (80.2)	98.0 ± 11.92 ^{b} (75.2)	77.5 ± 6.96 ^{bc} (73.0)
7	107.4 ± 8.88 ^{c} (87.5)	98.2 ± 3.49 ^{bc} (82.1)	114.9 ± 11.60 ^{abc} (88.2)	76.6 ± 3.37 ^{bc} (71.4)	105.0 ± 13.67 ^{abc} (87.2)	91.4 ± 6.45 ^{cd} (83.9)	96.6 ± 14.43 ^{b} (74.2)	84.7 ± 12.76 ^{bc} (80.7)	106.2 ± 10.39 ^{c} (87.3)	94.8 ± 5.94 ^{cd} (83.0)	105.7 ± 15.42 ^{b} (81.1)	80.7 ± 9.45 ^{bc} (76.1)
8	111.1 ± 3.37 ^{bc} (90.6)	101.6 ± 11.23 ^{abc} (85.0)	116.1 ± 20.93 ^{abc} (89.1)	81.1 ± 1.48 ^{b} (75.6)	108.4 ± 3.02 ^{abc} (89.9)	95.4 ±3.36 ^{bcd} (87.7)	97.1 ± 5.21 ^{b} (74.6)	90.0 ± 16.27 ^{abc} (85.8)	109.7 ± 3.23 ^{bc} (89.6)	98.5 ± 8.16 ^{bc} (86.2	106.6 ± 17.13 ^{b} (81.8)	85.5 ± 11.44 ^{b} (80.6)
Carotenoids [μg·g^{-1}FW] (% Control)												
Combination	**First Season**				**Second Season**				**SynthesisFirst—Second Season**			
	Tillering	**Stem Elongation**	**Ear Emergence**	**Flowering**	**Tillering**	**Stem Elongation**	**Ear Emergence**	**Flowering**	**Tillering**	**Stem Elongation**	**Ear Emergence**	**Flowering**
1	44.3 ± 3.93 ^{ab} (100)	42.8 ± 3.56 ^{a} (100)	47.7 ± 4.37 ^{ab} (100)	37.4 ± 2.24 ^{ab} (100)	40.6 ± 2.48 ^{ab} (100)	39.8 ± 8.61 ^{a} (100)	36.8 ± 3.94 ^{a} (100)	30.9 ± 6.47 ^{ab} (100)	42.5 ± 3.57 ^{abc} (100)	41.3 ± 6.13 ^{a} (100)	42.2 ± 7.03 ^{ab} (100)	34.2 ± 5.45 ^{ab} (100)
2	46.3 ± 1.90 ^{ab} (104.6)	45.5 ± 1.65 ^{a} (106.3)	49.9 ± 1.62 ^{a} (104.6)	39.6 ± 5.57 ^{a} (105.6)	42.3 ± 6.49 ^{ab} (104.2)	40.1 ± 2.49 ^{a} (100.7)	38.0 ± 2.32 ^{a} (103.3)	32.4 ± 5.01 ^{ab} (104.8)	44.3 ±4.81 ^{abc} (104.2)	42.8 ± 3.53 ^{a} (103.6)	43.9 ± 6.75 ^{a} (104.0)	35.9 ± 5.62 ^{a} (104.9)
3	47.9 ± 1.83 ^{a} (108.0)	44.1 ± 1.96 ^{a} (102.9)	37.9 ± 4.01 ^{ab} (101.3)	37.4 ± 9.11 ^{ab} (101.6)	43.6 ± 3.23 ^{ab} (107.4)	40.6 ± 8.91 ^{a} (101.9)	38.3 ± 2.53 ^{a} (104.1)	31.6 ± 3.91 ^{ab} (102.4)	45.7 ± 3.30 ^{ab} (107.5)	42.3 ± 6.07 ^{a} (102.4)	43.4 ± 8.15 ^{ab} (102.8)	34.8 ± 6.18 ^{ab} (101.7)
4	47.6 ± 1.12 ^{a} (107.4)	44.4 ± 3.35 ^{a} (103.6)	48.5 ± 2.32 ^{ab} (101.7)	38.4 ± 3.39 ^{a} (102.7)	44.8 ± 4.26 ^{a} (110.3)	41.8 ± 5.24 ^{a} (105.1)	38.0 ± 2.20 ^{a} (103.4)	34.2 ± 2.63 ^{a} (110.7)	46.2 ± 3.18 ^{a} (108.7)	43.1 ± 4.17 ^{a} (104.3)	43.3 ± 6.07 ^{ab} (102.6)	36.3 ± 4.94 ^{a} (106.1)
5	34.9 ± 2.48 ^{c} (78.9)	29.5 ± 3.03 ^{bc} (68.9)	33.8 ± 7.94 ^{b} (70.9)	25.8 ± 5.74 ^{c} (68.8)	31.1 ± 4.09 ^{b} (76.5)	29.1 ± 2.51 ^{b} (73.2)	26.3 ± 3.06 ^{b} (71.5)	22.9 ± 1.57 ^{b} (74.1)	33.0 ± 3.70 ^{e} (77.6)	29.3 ± 2.49 ^{b} (70.2)	30.1 ± 6.07 ^{c} (71.3)	24.4 ± 3.57 ^{c} (71.3)
6	38.8 ± 3.02 ^{bc} (87.6)	30.1 ± 5.21 ^{bc} (70.3)	37.7 ± 2.98 ^{ab} (79.1)	26.8 ± 2.71 ^{c} (71.6)	33.6 ± 7.21 ^{ab} (82.8)	34.6 ± 6.62 ^{ab} (86.9)	31.9 ± 4.46 ^{ab} (86.8)	25.9 ± 3.65 ^{ab} (83.9)	36.2 ± 5.71 ^{de} (85.1)	32.4 ± 5.87 ^{b} (78.4)	34.9 ± 4.62 ^{bc} (82.7)	26.4 ± 3.09 ^{c} (77.2)
7	40.2 ± 4.28 ^{abc} (90.7)	32.7 ± 2.49 ^{b} (76.4)	42.1 ± 5.52 ^{ab} (88.2)	29.3 ± 1.89 ^{abc} (78.3)	35.2 ± 2.98 ^{ab} (86.6)	36.0 ± 4.02 ^{ab} (90.6)	34.7 ± 2.43 ^{ab} (94.3)	27.7 ± 3.45 ^{ab} (89.6)	37.7 ± 4.29 ^{cde} (88.7)	34.4 ± 3.51 ^{ab} (83.3)	38.3 ± 5.54 ^{abc} (90.7	28.5 ± 2.61 ^{bc} (83.3)
8	40.6 ± 2.87 ^{abc} (91.6)	33.5 ± 2.79 ^{b} (78.2)	40.4 ± 5.45 ^{ab} (84.8)	27.8 ± 2.06 ^{bc} (74.4)	37.2 ± 3.09 ^{ab} (91.7)	36.4 ± 4.87 ^{ab} (91.5)	34.6 ± 5.79 ^{ab} (94.1)	27.5 ± 2.21 ^{ab} (89.2)	38.9 ± 3.24 ^{cde} (91.5)	34.9 ± 3.89 ^{ab} (84.5)	37.5 ± 5.96 ^{abc} (88.9)	27.7 ± 1.91 ^{bc} (81.0)

(1) Control, (2) 100 μM Vit PP soaking seeds, (3) 100 μM Vit PP foliar application, (4) 100 μM Vit PP soil application, (5) 1 mM $Pb(NO_3)_2$, (6) 1 mM $Pb(NO_3)_2$ + 100 μM Vit PP soaking seeds, (7) 1 mM $Pb(NO_3)_2$ + 100 μM Vit PP foliar application, (8) 1 mM $Pb(NO_3)_2$ + 100 μM Vit PP soil application; a–e—homogeneous groups.

4. Discussion

Lead is a significant environmental stressor that causes considerable damage to plant growth and development, hindering the advancement of sustainable agricultural practices. The World Health Organization recommends that the concentration of lead in soil should not exceed 85 mg kg^{-1} [53]. In this study, the dose of lead used, 1 mM $Pb(NO_3)_2$, equivalent to 207 mg kg^{-1} soil, significantly reduced only the length of roots and shoots in barley var. Eunova compared to the control. Differences in root growth are likely to be attributed to variations in lead uptake by cell walls and its accumulation in these organs, with lead accumulation in plant roots reaching as high as 95% [11,54,55].

4.1. Morphological Parameters

Literature reports indicate a decrease in morphological parameters under the influence of varying lead concentrations in wheat [34], beans [56], and tomatoes [57]. Our research [35,36] conducted on barley using the same lead dose showed a much more pronounced decrease in all investigated morphological parameters compared to this study. In studies conducted on MS media in vitro culture [35], there was a 46% decrease in root length, 59% decrease in seedling length, and 43% decrease in fresh weight compared to the control while, in experiments conducted on Petri dishes [36], the decreases were 81.8%, 36.6%, and 41.2%, respectively.

According to Hossain [58], plant responses and mechanisms to stress differ between hydroponic systems and field experiments.

Lead can bind to various soil components, such as clay minerals and organic matter, forming different complexes. However, only a small fraction of the lead in these complexes is available to plants [59].

Roots are the sole plant organs directly exposed to soil lead, likely contributing to the pronounced response observed in the barley var. Eunova studied in this experiment. The reduction in root growth may stem from disruptions in the physiological functions performed by roots, including the mobilization and circulation of elements from mineral and organic compounds in the soil, water uptake, and nutrient transport to other organs [5,60]. According to Aslam [14], lead ions can have variable effects on the absorption of important nutrients, potentially increasing the uptake of calcium and potassium while inhibiting magnesium. In addition, Dong et al. [61] suggest that elevated lead concentrations in soil substrates reduce the availability of other essential nutrients, such as iron, manganese, phosphorus, and zinc.

The reduced availability of these nutrients in cereal crops can have several adverse effects, including limiting the plants ability to produce ATP and adversely affecting the levels of enzymes responsible for all biochemical processes. Consequently, this could lead to reduced growth and yield [5].

The exogenous application of nicotinamide (100 µM) had a positive impact on root and shoot length (Figure 2). These results are consistent with the findings of Vendruscolo et al. [39], where the exogenous application of nicotinamide at doses of 100–300 mg L^{-1}, regardless of the application method (seed imbibition or foliar spraying), had a positive effect on the growth and yield of upland rice plants. In addition, a study by de Lima et al. [62] found that applying nicotinamide at concentrations ranging from 237.8 to 373.8 mg L^{-1} promoted growth and yield in soybean.

The improvement in plant growth characteristics following the exogenous application of this vitamin may result from increased energy reserves and nutrients, including carbohydrates [4,39,63,64], as reported by de Lima et al. [62], who reported that nicotinamide acts as a biostimulant. It serves as the primary precursor of nicotinamide adenine dinucleotide and its phosphate (NADH and NADPH), crucial coenzymes essential for ATP production and involved in oxidation-reduction reactions [65]. Niacin is vital for maintaining cellular metabolism, responsible for producing proteins, enzymes, carbohydrates, and lipids [66]. Furthermore, Hathout [67] demonstrated that soaking seeds in nicotinamide affected the

growth of endogenous phytohormones, such as gibberellic acid and indoleacetic acid, which play a pivotal role in regulating plant growth and development.

The use of vitamin PP in this study, through seed soaking, foliar spraying, and watering, mitigated the deleterious effects of lead salts and improved root and shoot growth in combinations grown with lead. These findings align with those of other authors, who used vitamin PP to alleviate stress from factors other than lead, such as salt stress [4,65,68,69] drought [20], or water stress [21,70].

In this study, it was demonstrated that the most effective stress-relieving impact on morphological parameters was achieved through the application of vitamin PP via foliar sprays. Vendruscolo et al. [39] found that both methods of nicotinamide application (soaking and spraying) had similar effects on plant growth. However, in a drought stress experiment conducted by Khurshid et al. [70], two methods of applying vitamin PP were compared: spraying and fertigation. Different amounts of nicotinamide were used in both methods, with spraying ranging from 0.737 to 2.215 g L^{-1} and fertigation from 0.492 to 1.477 g L^{-1}. Spraying proved more effective in alleviating drought stress on growth parameters.

The action of lead results in the generation of ROS. Their toxic effect involves excessive reactivity with various cellular components, such as proteins, sugars, lipids, and nucleic acids. As a result, various antioxidant systems (both enzymatic and nonenzymatic) are activated, leading to the production of substances indicating stress known as oxidative stress markers. CAT and POX are important antioxidant enzymes that function within cells to prevent the accumulation of excess ROS, specifically by detoxifying H_2O_2 [71]. ROS can induce lipid peroxidation in plants by oxidizing fatty acids present in cell membranes, leading to structural and functional damage. The extent of lipid peroxidation in plants can be evaluated through various methods, including the measurement of the volatile dialdehyde MDA content [72]. An increase in MDA within plant cells is an important indicator of oxidative stress [34,72,73]. In addition, Pro serves as a valuable indicator of stress intensity and acts as a nonenzymatic antioxidant, effectively counteracting the detrimental effects of ROS [71].

4.2. Biochemical Parameters

In this study, the biochemical response of barley var. Eunova was assessed by measuring the activity of antioxidant enzymes (CAT, POX), as well as the levels of Pro and MDA under abiotic stress conditions induced by the presence of 1 mM $Pb(NO_3)_2$ in the soil. The study revealed significant induction of enzymes such as CAT and POX, along with increased levels of Pro and MDA in plants growing in lead-contaminated soil compared to control plants. Similar results were reported by Dey et al. [74] for POX and by Jiang [75] for POX, Yang et al. [76] for CAT, POX, MDA, Alamri et al. [34] for CAT, MDA, Sędzik et al. [35] for CAT, POX, Pro, MDA, Cândido et al. [77] for CAT, MDA, Pirzadoh et al. [78] for Pro, Navabpaur et al. [73] for CAT, MDA, Khan et al. [13] for CAT, POX, Pro, Ahmad et al. [33] for Pro, Sędzik-Wójcikowska et al. [36] for CAT, POX, Pro, MDA. However, conflicting results were presented by Verma and Dubey [55], Dey et al. [74] for CAT, Sędzik et al. [35] for CAT at the highest lead dose, and Li et al. [79] for CAT and POX. However, conflicting results were presented by Verma and Dubey [55], Dey et al. [74] for CAT, Sędzik et al. [35] for CAT at the highest lead dose, and Li et al. [79] for CAT and POX. The varied responses of these enzymes under similar stress conditions reported by several authors may stem from not entirely identical experimental conditions. An increase in the activity of these enzymes indicates oxidative stress in cells. A significant decrease in CAT and POX activity in plants, as reported by Dey et al. [74], suggests a weakening of the systems for scavenging ROS generated in stressful situations. These authors propose that the decrease in enzyme activity could result from enzyme inhibition, given that these proteins are highly sensitive to a number of factors. The diverse responses in enzyme activity also depend on factors such as the plant species, the specific stress factor applied, its concentration, and the duration of exposure [80].

An increase in MDA levels indicates damage to cell membranes. This damage can result in reduced water absorption from the environment, decreased conductance, leading to reduced turgor, and a limitation of transpiration [81]. According to Dey et al. [74], the following membrane properties are altered when damaged: fluidity, permeability, rate, and selectivity of nutrient transport. Research conducted by Özturk and Demir [82] has shown that Pro plays an important role in plant responses to heavy metals, probably related to its antioxidant properties, metal-chelating function, and ability to protect enzymes such as CAT and POX. Pro is also involved in stabilizing membranes, proteins, and DNA [83].

The application of vitamin PP through seed soaking, foliar spraying, and irrigation in this study alleviated the deleterious effects of lead salts and improved the measured biochemical parameters in combinations growing with lead (Tables 1 and 2). Exogenously applied vitamin PP reduced the activity of antioxidant enzymes (CAT and POX) and the levels of MDA and Pro in plants growing with lead. The most effective relief from lead stress was observed with foliar spraying and irrigation using vitamin PP. Our findings align with those of other authors, who used vitamin PP under stress conditions other than lead, such as salt stress [63], drought stress [20], and water stress [21]. In the study by El-Bassiouny et al. [63], the application of vitamin PP through seed imbibition and foliar spray reduced the effects of salinity stress by decreasing the Pro content in plants exposed to salinity stress, with the foliar spray having a greater effect on Pro. Meanwhile, in the study by El-Bassiouny et al. [21], the exogenous application of nicotinamide, in addition to mycorrhizal supplementation under water stress conditions, decreased the MDA content while increasing the activity of CAT and POX. The decrease in the activity of antioxidant enzymes (CAT and POX) and the content of MDA and Pro in lead-grown plants following the introduction of exogenous vitamin PP may be attributed to vitamin PP reducing ROS levels by enhancing both enzymatic and nonenzymatic antioxidant defense mechanisms, as well as the production of osmotically active substances [22].

4.3. Physiological Parameters

Chloroplasts are cell organelles susceptible to the production of ROS.

In this study, the physiological response of barley var. Eunova was determined by measuring the total chlorophyll and carotenoid content under abiotic stress conditions induced by the presence of 1 mM $Pb(NO_3)_2$ in the soil. The results demonstrated a decrease in pigment content in plants growing in lead-contaminated soil compared to control plants. Similarly, other studies have shown that lead decreases pigment content compared to controls [13,33,35,36,78,84]. The reduced chlorophyll content under the influence of lead may be attributed to its inhibition of magnesium availability. Magnesium occupies a central position in the chlorophyll molecule, and its presence is necessary for the proper functioning of this compound and the process of photosynthesis [14].

Exogenously applied vitamin PP, used in this study through irrigation, alleviated the harmful effects of lead salts and increased the pigment content in plants grown in the presence of lead. Similar outcomes have been reported by other authors using vitamin PP in response to stressors other than lead, such as salt stress [4,63,65,69] and drought stress [20]. The observed increase in chlorophyll content due to exogenously applied vitamin PP could be attributed to the activation of enzymes involved in regulating photosynthetic carbon reduction [85]. Vendruscolo and Seleguin [7] propose that this vitamin provides an antioxidant defense to the photosystem cells responsible for converting light energy into assimilates used in carboxylation processes, thereby influencing plant development and providing effective protection against stressors.

5. Conclusions

The application of 1 mM $Pb(NO_3)_2$ resulted in a decrease in root and shoot length, along with increased catalase and peroxidase activity, as well as the content of malondialdehyde, proline, and assimilatory pigments during developmental stages in which spring barley var. Eunova was examined in a pot experiment. Exogenous vitamin PP had a signifi-

cant and favorable effect on the morphological, biochemical, and physiological parameters studied, thereby reducing the toxicity of lead salts. The effect of the applied dose of vitamin PP depends on the form of application. The most effective reduction in lead stress was achieved through foliar spraying and watering with vitamin PP. This research suggests the potential use of vitamin PP to enhance plant resistance to lead stress. However, field studies are necessary to ensure the practical relevance of vitamin PP application methods in agriculture.

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10. Pisemne oświadczenia współautorów publikacji naukowych stanowiących rozprawę doktorską

OŚWIADCZENIA

o wkładzie autora w publikację

Publikacja 1: Sędzik M., Smolik B., Krupa-Małkiewicz M. 2015. Effect of lead on germination and some morphological and physiological parameters of 10-day-old seedlings of various plant species. Ochrona Środowiska I Zasobów Naturalnych 26(3): 22-27.

Oświadczam, że mój udział w pracy polegał na współtworzeniu koncepcji, założeniu i przeprowadzeniu doświadczenia, a także wykonaniu analiz laboratoryjnych. Ponadto opracowanie i interpretacja wyników, pisanie publikacji oraz jej korekta.

Wkład mojej pracy w w/w publikację oceniam na 60%. Podpis: Sędzik-Wójcikowska

Wkład pozostałych współautorów:

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Publikacja 2: Sędzik-Wójcikowska M., Smolik B., Krupa-Małkiewicz M 2019. Effect of nicotinamide in alleviating stress caused by lead in spring barley seedling. Journal of Elementology 24(1): 281-291.

Oświadczam, że mój udział w pracy polegał na współtworzeniu koncepcji, wyborze metodyki badań, założeniu i przeprowadzeniu doświadczenia, a także wykonaniu analiz laboratoryjnych. Ponadto opracowanie i interpretacja wyników, pisanie publikacji oraz jej korekta.

Wkład mojej pracy w w/w publikację oceniam na 60%. Podpis: Sędzik-Wójcikowska

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Publikacja 3: **Sędzik-Wójcikowska M.**, Krupa-Małkiewicz M., Smolik B. 2023. The Effect of Use of the Biologically Active Substances in Alleviating the Stress Caused by Lead in Barley Seedling on the Basis of Biochemical and Physiological Parameters. Journal of Ecological Engineering 24(8).

Oświadczam, że mój udział w pracy polegał na współtworzeniu koncepcji, wyborze metodyki badań, założeniu i przeprowadzeniu doświadczenia, a także wykonaniu analiz laboratoryjnych. Ponadto opracowanie i interpretacja wyników, pisanie publikacji oraz jej korekta.

Wkład mojej pracy w w/w publikację oceniam na 55%. Podpis: Sędzik-Wójcikowska

Wkład pozostałych współautorów:

Smolik Beata – 25%. Podpis: Beata Smolik

Krupa-Małkiewicz Marcelina – 20%. Podpis: MKM

Publikacja 4: Smolik B., **Sędzik-Wójcikowska M.**, 2024 Effect of Application Methods of Nicotinamide on the Alleviation of Lead-Induced Stress in Spring Barley. Agronomy 14(6): 1314

Oświadczam, że mój udział w pracy polegał na współtworzeniu koncepcji, wyborze metodyki badań, przeprowadzeniu doświadczenia wazonowego i wykonaniu analiz laboratoryjnych. Ponadto opracowanie wyników oraz współtworzenie publikacji.

Wkład mojej pracy w w/w publikację oceniam na 45%. Podpis: Sędzik-Wójcikowska

Wkład pozostałych współautorów:

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