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Wpływ typu gleb i pierwiastków na kiełkowanie i wzrost roślin piaszczystych muraw bezwapiennych i kserotermicznych muraw wapieniolubnych

The influence of soil types and elements
on germination and growth of plants
from non-calcareous psammophilous grasslands
and calcareous xerothermic grasslands

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2. Wykaz artykułów naukowych wchodzących w skład rozprawy doktorskiej

P–1:

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Praca oryginalna; IF_{2020}: 6,114; $MEiN_{2020}$: 200

P–2:

Wala, M.*, Kołodziejek, J., Mazur, J., 2023. The diversity of iron acquisition strategies of calcifuge plant species from dry acidic grasslands. *Journal of Plant Physiology* **280**, 153898. DOI: 10.1016/j.jplph.2022.153898

Praca oryginalna; IF_{2021}: 3,686; $MEiN_{2023}$: 100

P–3:

Wala, M.*, Kołodziejek, J., Mazur, J., Cienkowska, A., 2021. Reactions of two xeric-congeneric species of *Centaurea* (Asteraceae) to soils with different pH values and iron availability. *PeerJ* **9**, e12417. DOI: 10.7717/peerj.12417

Praca oryginalna; IF_{2021}: 3,061; $MEiN_{2021}$: 100

P–4:

Wala, M.*, Kołodziejek, J., Wilk, T., 2022. Acidity and availability of aluminum, iron and manganese as factors affecting germination in European acidic dry and alkaline xerothermic grasslands. *PeerJ* **10**, e13255. DOI: 10.7717/peerj.13255

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Artykuły naukowe:

Kołodziejek, J., Patykowski, J., **Wala, M.*,** 2017. Effect of light, gibberellic acid and nitrogen source on germination of eight taxa from dissapearing European temperate forest, *Potentillo albae Quercetum*. *Scientific Reports* **7**, 13924. DOI: 10.1038/s41598-017-13101-z

Kołodziejek, J., Patykowski, J., **Wala, M.*,** 2018. An experimental comparison of germination ecology and its implication for conservation of selected rare and endangered species of *Dianthus* (Caryophyllaceae). *Botany* **96**, 319–328. DOI: 10.1139/cjb-2017-0213

Rusek, P., **Wala, M.,** Druszczyńska, M., Fol, M., 2018. Infectious agents as stimuli of trained innate immunity. *International Journal of Molecular Sciences* **19**, 456. DOI: 10.3390/ijms19020456

Patykowski, J., Kołodziejek, J., **Wala, M.*,** 2018. Biochemical and growth responses of silver maple (*Acer saccharinum* L.) to sodium chloride and calcium chloride. *PeerJ* **6**, e5958. DOI: 10.7717/peerj.5958

Kołodziejek, J., Patykowski, J., **Wala, M.*,** 2019. Dormancy, germination, and sensitivity to salinity stress in five species of *Potentilla* (Rosaceae). *Botany* **97**, 452–462. DOI: 10.1139/cjb-2019-0038

Wala, M.*, Kołodziejek, J., Patykowski, J., 2021. Nitrogen signals and their ecological significance for seed germination of ten psammophilous plant species from European dry acidic grasslands. *PLoS One* **16**, e0244737. DOI: 10.1371/journal.pone.0244737

Skwarek, M., **Wala, M.,** Kołodziejek, J., Sieczyńska, K., Lasoń-Rydel, M., Ławińska, K., Obraniak, A., 2021. Seed coating with biowaste materials and biocides – environment-friendly biostimulation or threat? *Agronomy* **11**, 1034. DOI: 10.3390/agronomy11061034

Wala, M.*, Skwarek-Fadecka, M., Kołodziejek, J., Mazur, J., Lasoń-Rydel, M., Krępska, M., 2022. Effect of the Fe-HBED chelate on the nutritional quality of tomato fruits. *Scientia Horticulturae* **293**, 110670. DOI: 10.1016/j.scienta.2021.110670

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4. Streszczenie

Niedobory żelaza stanowią jedno z głównych źródeł ograniczeń, jakich rośliny doświadczają na glebach zasadowych. Pomimo licznych prac, etiologia tychże niedoborów (objawiających się zazwyczaj w postaci chloroz międzynerwowych) jest niejasna, co przyczyniło się do utrwalenia pojęcia tzw. „paradoksu chlorozy zależnej od żelaza". Ponadto, podatność na niedobory żelaza oraz ich przyczyny i skutki dla roślin występujących w obrębie piaszczystych muraw bezwapiennych oraz kserotermicznych muraw wapieniolubnych (skrajnie różnych pod względem odczynu i dostępności żelaza) pozostawały w znaczącej mierze niezbadane. Uzasadniało to potrzebę podjęcia dalszych badań w tym zakresie. W pracy zbadano łącznie 24 gatunki roślin naczyniowych. Wyniki pierwszego etapu badań nad roślinami kalcyfilnymi (opisane w pracy P–1) wyraźne wskazały, że, podczas gdy *Salvia verticillata* i *Veronica teucrium* są gatunkami niewykazującymi objawów niedoboru żelaza, *Aster amellus*, *Betonica officinalis* oraz *Prunella grandiflora* należą do grupy roślin, w których rozwój chlorozy zależnej od żelaza ma najczęściej złożoną etiologię i pociąga za sobą liczne negatywne skutki fizjologiczne. Dane zebrane podczas drugiego etapu badań (opisane w pracy P–2) wskazały zróżnicowanie podatności na niedobory żelaza również w obrębie roślin zaliczanych do kalcyfobów, różnicując badane gatunki na podatne (*Antennaria dioica* i *Jasione montana*) oraz odporne (*Alyssum monatnum*, *Hypochaeris radicata* i *Potentilla arenaria*) na niedobory żelaza. W tym przypadku chloroza również miała złożoną przyczynę i pociągała za sobą negatywne skutki na poziomie funkcjonalnym. Dalsze badania nad gatunkami kongenerycznymi, występującymi w obrębie obu typów muraw (*Centaurea scabiosa* i *C. stoebe*) wskazały, że nisze ekologiczne gatunków mających szerokie wymagania względem odczynu gleby różnicowane są na poziomie indywidualnego zapotrzebowania na żelazo (wyniki przestawione w pracy P–3). W ogólnym ujęciu wyniki wskazały rozłączność wymagań badanych gatunków względem odczynu gleby oraz dostępności żelaza. Stwierdzono również brak zależności między preferencjami substratowymi, a tolerancją na niedobory tegoż pierwiastka. Ponadto, pomimo ewidentnego udziału manganu i cynku w kształtowaniu się niedoborów żelaza, etiologia tego ograniczenia jest specyficzna gatunkowo, przez co należy ją traktować jako niedobór zróżnicowany oraz złożony. Ostatni etap prac nad roślinami występującymi w obrębie badanych muraw (praca P–4) wskazał, że odczyn oraz dostępność żelaza, manganu i glinu mogą pełnić rolę filtrów selekcyjnych również na wczesnych etapach ontogenezy, wpływając na zdolność nasion do wykiełkowania. Stwierdzono również, że żelazo i mangan pełnią rolę drugorzędną w stosunku do odczynu i glinu. Co więcej, tolerancja względem tychże czynników jest szersza w fazie kiełkowania niż na dalszych etapach rozwoju osobniczego i nie zawsze pokrywa się szacunkami odnośnie centrów występowania badanych gatunków. W świetle uzyskanych wyników stwierdzono daleko posunięte zróżnicowanie wymagań roślin występujących naturalnie, zarówno w obrębie piaszczystych muraw bezwapiennych, jak i kserotermicznych muraw wapieniolubnych, co podkreśla potrzebę rozpatrywania ekologii zbiorowisk wielogatunkowych przez pryzmat ekologii gatunków, które je budują.

5. Abstract

One of the main limitations experienced by plants occurring on alkaline soils is iron deficiency. Despite numerous works, the etiology of these deficiencies (usually manifesting as interveinal chlorosis) remains unclear, thus giving rise to the "iron-dependent chlorosis paradox." The present study examines the manifestation of iron deficiency in vascular plants from non-calcareous psammophilous grasslands and calcareous xerothermic grasslands, two environments which differ greatly in terms of soil reaction and iron availability, as well as its causes and effects. In total, 24 species of vascular plants were studied. The results of the first part of the study regarding calciphilous plants (described in article P–1) clearly indicate that while *Salvia verticillata* and *Veronica teucrium* do not show iron deficiency symptoms, *Aster amellus*, *Betonica officinalis* and *Prunella grandiflora* demonstrate a complex etiology of iron-dependent chlorosis that entails numerous negative physiological effects. Data collected during the second stage (described in article P–2) indicate that the species classified as calciphobes demonstrated varying susceptibility to iron deficiency, thus differentiating them as susceptible (*Antennaria dioica* and *Jasione montana*) or resistant (*Alyssum monatnum*, *Hypochaeris radicata* and *Potentilla arenaria*) to iron deficiency. Additionally, in this case, the chlorosis was of a complex cause and triggered negative effects at the functional level. Further studies on congeneric species found within both types of grassland (*Centaurea scabiosa* and *C. stoebe*) indicated that the ecological niches of species characterized by broad tolerance to soil pH were differentiated at the level of individual iron requirements (results presented in article P–3). Overall, the results indicated no correlation between the requirements for soil pH and iron availability, nor between substrate preference and tolerance to iron deficiencies. Moreover, although manganese and zinc clearly contribute to the development of iron deficiency, the limitation nevertheless demonstrates a species-specific etiology, making this a diverse and complex issue. The latest stage of the presented research on plants that can be found within the studied grasslands (article P–4) indicated that the soil reaction and availability of iron, manganese and aluminum can act as selection filters at early ontogenetic stages, affecting the ability of seeds to complete germination. However, iron and manganese were found to play a secondary role when compared to pH and aluminum. Additionally, the plants demonstrate greater tolerance to the studied factors during germination than during further stages of ontogenesis, and this does not always fit the estimated centers of abundance of the studied species. Hence, the environmental requirements of plants occurring naturally within both non calcareous psammophilous grasslands and calcareous xerothermic grasslands appear to demonstrate considerable differentiation. Therefore, it should be emphasized that the ecology of multispecies assemblages can only be interpreted through the prism of the ecology of the individual species occurring within them.

6. Wprowadzenie

Murawy to naturalne lub półnaturalne, pierwotne lub wtórne, nieleśne zbiorowiska roślinne z dominacją traw, występujące na podłożu mineralnym, w których pokrywa roślinna jest stosunkowo gęsta, a ruń jest niższa niż w obrębie łąk (przede wszystkim ze względu na niższą dostępność wody oraz niską zasobność gleb; Peeters, 2009; Sanderson i wsp., 2009; Dengler i wsp., 2014; Roo-Zielińska, 2014; Feurdean i wsp., 2018).

Najogólniejszy podział naturalnych muraw palearktycznych przebiega w oparciu o ich relację względem warunków siedliskowych oraz o stosunek lokalny do zbiorowisk leśnych (Dengler i wsp., 2014). Wobec powyższego, wyróżnia się stepy lub murawy nawiązujące do stepów (na siedliskach zbyt suchych dla rozwoju lasów), murawy alpejskie (na siedliskach zbyt zimnych dla rozwoju lasów) oraz murawy ekstrazonalne i azonalne (w których warunki lokalno-siedliskowe w obrębie biomów leśnych uniemożliwiają wzrost drzew; Dengler i wsp., 2014; Török i wsp., 2018). Występowanie muraw o charakterze półnaturalnym uzależnione jest ściśle od regularnych, antropogenicznych zaburzeń siedliska (Dengler i wsp., 2014; Feurdean i wsp., 2018).

Wyraźnie zarysowana zmienność muraw europejskich znajduje odzwierciedlenie w znacznej i ciągle rosnącej ilości wyróżnianych w syntaksonomii zespołów i związków (Török i wsp., 2018). Wśród muraw suchych i ciepłych (kserotermicznych) wyróżnia się w randze klas dwa kontrastujące ze sobą pod względem czynników edaficznych typy muraw: piaszczyste murawy bezwapienne (*Koelerio-Corynephoretea canescentis* Klika in Klika et Novák 1941) oraz kserotermiczne murawy wapieniolubne (*Festuco-Brometea* Br.-Bl. et Tx. ex Soó 1947; Mucina i wsp., 2016). Pomimo licznych prac, pozycja syntaksonomiczna obu typów muraw, ich wewnętrzne zróżnicowanie oraz wzajemne relacje nadal pozostają elementem dyskusji naukowej (Mucina i wsp., 2016; Willner i wsp., 2019).

Syntaksonomiczny podział na piaszczyste murawy bezwapienne i kserotermiczne murawy wapieniolubne odpowiada stosunkowo wiernie bimodalnemu rozkładowi odczynu gleb mineralnych na których zbiorowiska te występują (Bothe, 2015; Leuchner i Ellenberg, 2017), tj. piaszczyste murawy bezwapienne (z klasy *Koelerio-Corynephoretea canescentis*) zasiedlają gleby kwaśne i bezwapienne, a kserotermiczne murawy wapieniolubne (z klasy *Festuco-Brometea*) zasiedlają zasadowe gleby wapienne (Matuszkiewiecz, 2022). Wyjątek stanowią kontynentalne murawy piaszczyste ze związku *Koelerion glaucae* (Volk 1931) Klika 1935 (*Koelerio-Corynephoretea canescentis*), które florystycznie nawiązują wprost do wapieniolubnych muraw kontynentalno-przyśródziemnomorskich z rzędu *Festucetalia valesiacae* Br.-Bl. et R.Tx 1943 (*Festuco-Brometea*; dominujące w Europie Wschodniej; Matuszkiewicz, 2022). Zacieranie się różnic między nimi stanowi źródło kontrowersji w syntaksonomii muraw (Matuszkiewicz, 2022), a część ujęć (szczególnie spojrzenie ekologiczne) sugeruje wręcz brak wyraźnych granic między tymiż fitocenozami, a więc stosunkowo płynne przechodzenie jednych muraw w drugie w ciągłym spektrum wymagań gatunków względem odczynu gleby (Schulze i wsp., 2019).

Kwaśne i oligotroficzne gleby, na których spotyka się piaszczyste murawy bezwapienne (Czyżewska, 1992; Jentsch i Beyschlag, 2003), klasyfikowane są najczęściej jako gleby bielicoziemne (dla których akumulacja związków żelaza i glinu oraz próchnicy w poziomach podpowierzchniowych odgrywa rolę diagnostyczną; ang. *Podzols*); rzadziej murawy te rozwijają się na podłożu gleb piaszczystych o słabo rozwiniętym lub nierozwiniętym profilu (arenosole; ang. *Arenosols*) lub na glebach inicjalnych bez wyraźnego rozwoju profilu (słabo ukształtowane gleby erozyjne; ang. *Regosols*; IUSS WRB Working Group, 2014). Wszystkie typy gleb na których występują piaszczyste murawy bezwapienne zbudowane są z materiału bezwapiennego, głównie piasku krzemianowego pochodzenia eolicznego, glacjalnego lub aluwialnego (*Podzols* i *Arenosols*) lub nieskonsolidowanych osadów klastycznych (*Regosols*; IUSS WRB Working Group, 2014), a jeśli zawierają one znaczące ilości wapnia, to przyczyną są procesy wtórne, w tym antropogeniczne (np. wapnowanie; Holland i wsp., 2018). Niska wartość pH (ujemnego logarytmu dziesiętnego z aktywności jonów wodorowych) gleb kwaśnych warunkowana jest niską pojemnością sorpcyjną oraz (w mniejszym stopniu) działaniem buforu glinowego (dla wartości pH poniżej 4,2) i żelazowego (dla wartości pH poniżej 3,8; Ulrich and Sumner, 1991).

Kserotermiczne murawy wapieniolubne związane są z glebami zasadowymi lub obojętnymi, wywodzącymi się ze skał macierzystych bogatych w wapń (najczęściej w postaci węglanu wapnia – $CaCO_3$ – głównie w postaci kalcytu; znacznie rzadziej z dominacją lub udziałem siarczanów wapnia – $CaSO_4$ – w postaci gipsów lub węglanów wapnia i magnezu – $CaMg(CO_3)_2$ – w postaci dolomitu; Merunková i wsp., 2012; Dengler i wsp., 2014). Gleby na których występują kserotermiczne murawy wapieniolubne mają różną miąższość i cechują się zróżnicowaną zawartością części szkieletowych. Najczęściej są to płytkie, inicjalne lub słabo ukształtowane gleby szkieletowe (ang. *Leptosols*). Część muraw rozwija się również na glebach czarnoziemnych, czyli wysoce wysyconych kationami glebach cechujących się znaczną akumulacją materii organicznej w mineralnej warstwie powierzchniowej (ang. *Phaeozems*), glebach brunatnoziemnych o umiarkowanie rozwiniętym profilu (ang. *Cambisiols*) oraz glebach inicjalnych bez wyraźnego rozwoju profilu (*Regosols*; IUSS WRB Working Group, 2014). Cechą wspólną wszystkich tych gleb jest wysoka zawartość bogatej w wapń frakcji szkieletowych (określanej jako *calcaric* – zawierającej pierwotny $CaCO_3$, *gypsyric* – zawierający pierwotny $CaSO_4$ lub *dolomitic* – zawierający pierwotny $CaMg(CO_3)_2$, w zależności od chemizmu skały macierzystej; IUSS WRB Working Group, 2014). Rozważając wymienione typy gleb, kserotermiczne murawy wapieniolubne związane są najczęściej z rędzinami (ang. *Rendzinas* syn. *Rendzic Leptosols*) – płytkimi, silnie szkieletowymi glebami, których poziom diagnostyczny *mollic* zawiera części szkieletowe lub położony jest na skale macierzystej zawierającej więcej niż 40% $CaCO_3$ (IUSS WRB Working Group, 2014). Wysoka wartość pH wyżej wymienionych gleb zasadowych warunkowana jest obecnością $CaCO_3$ i działaniem buforu węglanowego (w zakresie wartości pH 8,6–6,2; Ulrich and Sumner, 1991); gleby uboższe w $CaCO_3$ (np. wywodzące się z gipsu) podatne są na zakwaszenie (Leuchner i Ellenberg, 2017).

Wypadkowy odczyn gleby zależny jest m.in. od parametrów fizykochemicznych skał macierzystych oraz wywodzącej się od niej zwietrzeliny budującej fazę stałą gleby

(Vestin in wsp., 2006; Müller i wsp., 2022), struktury pionowej (miąższości i warstwowości; Tyler, 2004; Müller i wsp., 2022) oraz poziomej (zróżnicowania mozaikowego) gleby, przeszłych oraz teraźniejszych procesów glebotwórczych (w tym erozja i nanoszenie materiału allochtonicznego; Martignier i wsp., 2012) i formy użytkowania terenu przez człowieka (w tym wapnowanie i nawożenie; Holland i wsp., 2018; Müller i wsp., 2022). Ponadto, gleba w strefie ryzosferalnej jest silnie modyfikowana przez pokrywę roślinną (Strawn i wsp., 2020).

Odczyn gleby oraz dostępność wapnia nie są jedynymi cechami różnicującymi biotop obu typów muraw, gdyż wartość pH gleby mocno rzutuje na procesy biogeochemiczne kontrolujące dostępność pozostałych pierwiastków (Bothe, 2015; Strawn i wsp., 2020). Gleby kwaśne odznaczają się większą zawartością i dostępnością żelaza, manganu i glinu niż gleby zasadowe (Abedi i wsp., 2013; Bothe, 2015; Strawn i wsp., 2020), przy czym wzajemny stosunek ilościowy żelaza i manganu może być różny (Strawn i wsp., 2020). Zarówno gleby kwaśne, jak i zasadowe uznawane są za oligotroficzne pod względem zasobności w azot i fosfor, przy czym gleby kwaśne są uboższe niż gleby zasadowe (Leuchner i Ellenberg, 2017). Ponadto, istnieje wyraźne zróżnicowanie dostępnych mineralnych form azotu i fosforu: amonowa forma azotu ($NH4^{+}$) oraz nieco lepiej biodostępny diwodorofosforan (V) (HPO_4^{2-}) dominują w glebach kwaśnych, a azotanowa forma azotu (NO^{3-}) oraz nieco gorzej biodostępny wodorofosforan (V) (HPO_4^{2-}) w glebach zasadowych (Leuchner i Ellenberg, 2017; Strawn i wsp., 2020). Zawartość i dostępność pozostałych pierwiastków, w tym przede wszystkim magnezu, cynku, miedzi, boru, molibdenu i chloru również różnicuje gleby kwaśne od zasadowych (Strawn i wsp., 2020), jednakże nie została dobrze poznana w kontekście badań porównawczych nad murawami (Bothe, 2015).

Część suchych i ciepłych muraw występuje również na innych typach gleb. Na uwagę zasługują tu w szczególności: murawy związane z nieczęstymi w Europie Środkowej i Wschodniej, wywodzącymi się z gipsu (i przez to podatnymi na zakwaszenie), glebami bogatymi w wapń (Sarosiek, 1964; Łuszczyńska, 2006); zróżnicowane pod względem wymagań co do odczynu murawy związane z bogatymi w magnez, nikiel, chrom i kobalt glebami wywodzącymi się z serpentynitów (Żołnierz, L., 2007); oraz murawy galmanowe odznaczające się odczynem zasadowym i wysoką dostępnością wapnia, cynku oraz ołowiu (Szarek-Łukaszewska i Grodzińska, 2011).

Rośliny występujące w obrębie muraw można zaliczyć (w zależności od częstości i liczebności występowania) do grupy gatunków budujących (dominujących), satelitarnych (akcesorycznych) bądź pośrednich (influentnych; (Ozinga, 2008). Pomimo zdecydowanej przewagi ilości gatunków bylin niebędących trawami nad gatunkami traw w obrębie muraw, z definicji zbiorowiska te zdominowane są przez te ostatnie (Lauenroth I Adler, 2008). Trawy wieloletnie cechują się lepszymi zdolnościami przetrwania oraz lepszą oczekiwaną I realizowaną długością życia niż pozostałe byliny (Lauenroth I Adler, 2008). Ponadto, obie grupy roślin odznaczają się odmienną architekturą i różnymi (często przeciwstawnymi) mechanizmami tolerancji stresowych czynników środowiska (Tester i Bacic, 2005). Warto zaznaczyć, że choć znaczna część piaszczystych muraw

bezwapiennych i kserotermicznych muraw wapieniolubnych cechuje się wyraźnym bogactwem gatunkowym roślin innych niż trawy (Török i wsp., 2018), to spora część taksonów ma status roślin rzadkich lub zagrożonych (Kaźmierczakowa i wsp., 2014). Podnosi to rangę problemu nieznajomości szczegółów ich ekologii, zarówno w kontekście poznawczym, jak i aplikacyjnym.

Ze zróżnicowaniem taksonomicznym roślin występujących w obrębie muraw związane jest również zróżnicowanie ekologiczne, odnoszące się m.in. do tolerancji ekologicznej – podczas gdy część gatunków cechuje się szeroką amplitudą tolerancji ekologicznej (gatunki eurytopowe, generaliści), pozostała część wykazuje węższą amplitudę tolerancji, często o charakterze ściśle stenobiontycznym (gatunki stenotopowe; specjaliści; Gaujour i wsp., 2011). Gatunki występujące w obu typach muraw posiadają, jako ogół, szereg specjalistycznych adaptacji strukturalnych (anatomicznych i morfologicznych) oraz funkcjonalnych (fizjologicznych i ekologicznych) umożliwiających im przetrwanie w warunkach silnego oświetlenia oraz deficytu wody (Kooyers, 2015; Leuchner i Ellenberg, 2017). Uważa się, że część adaptacji do warunków kserotermicznych związana jest z żywieniem mineralnym: fizjonomia korzeni warunkuje dużą powierzchnię poboru wody i składników mineralnych, współczynnik wykorzystania wody (ang. *water use efficiency*, WUE) i przewodność szparkowa związane są z gospodarką makroelementami, a abscyzja uszkodzonych liści (zaburzających bilans gospodarki wodnej) zachodzi po odzysku substancji odżywczych z tychże organów (Leuchner i Ellenberg, 2017). Z drugiej strony, choć wszystkie gatunki występujące w obrębie ciepłych i suchych muraw posiadają cechy związane ze specjalizacją do siedlisk suchych i ciepłych, to można wśród nich wyróżnić zarówno gatunki będące generalistami, jak i specjalistami pod względem wymagań edaficznych (Cachovanová I wsp., 2012). Pod względem nomenklatorycznym przyjęło się, że rośliny, których wąskie optimum ekologiczne realizuje się w obrębie kserotermicznych muraw wapieniolubnych, nazywane są roślinami zasadolubnymi (wapieniolubnymi) – kalcyfilami (ang. *basophile* lub *calcicole*), a rośliny o wąskim optimum związanym z piaszczystymi murawami bezwapiennymi uznawane są za organizmy kwasolubne – kalcyfoby (ang. *acidophile* lub *calcifuge*; Bothe, 2015; Leuchner i Ellenberg, 2017). Wymienne użycie określeń odnoszących się do preferencji względem odczynu gleby i dostępności wapnia jest popularne i wydaje się być dla większości przypadków w pełni uzasadnione (Bothe, 2015). Problem mogą sprawiać jedynie rośliny klasyfikowane jako organizmy gipsofilne lub gipsoznośne, występujące wyłącznie na bogatych w wapń, choć mniej zasadowych (i podatnych na zakwaszenie) glebach wywodzących się z gipsów (Leuchner i Ellenberg, 2017). Warto przy tym podkreślić, że w Europie Środkowej i Wschodniej stenotopowe gatunki gipsofilne nie występują (lub ich preferencje są kwestionowane; Escudero i wsp., 2015; Leuchner i Ellenberg, 2017; Pérez-García, 2018), głównie ze względu na brak odpowiednich siedlisk w ewolucyjnej skali czasu.

Cechą wyróżniającą gatunki występujące w obrębie obu typów muraw względem pozostałych typów siedlisk jest zatem preferencja względem miejsc suchych i ciepłych przy jednoczesnych wyraźnie zarysowanych wymaganiach względem odczynu gleby (tzw. rośliny typu *bodenstet*; Kruckeberg, 1967) lub (rzadziej; Olsson i wsp., 2012) wyraźna kserotermofilność i brak wyraźnego zróżnicowania wymagań edaficznych (tzw. rośliny typu

bodenvag; Kruckeberg, 1967). Wysokie wymagania względem światła i temperatury odróżniają je zatem od roślin występujących na murawach alpejskich związanych z płytkimi, szkieletowych glebami mineralnymi. Co prawda murawy te tworzone przez równie głęboko wyspecjalizowane kalcyfoby (wysokogórskie murawy acidofilne z klasy *Juncetea trifidi* Hadač in Klika et Hadač 1944) oraz kalcyfile (wysokogórskie murawy nawapiene z klasy *Seslerietea variae* Br.-Bl. 1948 em. Oberd. 1978), ale obserwowane u nich przystosowania do specyficznych, wysokogórskich warunków świetlnych i termalnych sprawiają, że ich strategie życiowe są w dużej mierze nieporównywalne w sposób bezpośredni (Leuchner i Ellenberg, 2017; Rosbakh i Poschlod, 2021).

Każdy gatunek cechuje się odmienną, specyficzną dla niego odpornością na stres, w tym stres abiotyczny. Uważa się, że odporność ta zmienia się w ontogenezie i jest zależna od wieku: roślinne organizmy juwenilne różnią od osobników dojrzałych i starzejących się (Rankenberg i wsp., 2021). Jednakże, nadal stosunkowo niewiele wiadomo o zdolnościach roślin do radzenia sobie ze stresem na etapie przedwschodowym, tj. w trakcie kiełkowania. Uważa się, że rośliny wykazują międzygatunkowe zróżnicowanie strategii kiełkowania i przez to również inne amplitudy tolerancji względem głównych czynników regulujących ten proces (światło, temperatura oraz dostępność wody; Baskin i Baskin, 2014), jak i drugorzędnych czynników modulujących, w tym tych związanych z matrycą gleby (odczyn, dostępność pierwiastków). Choć wpływ odczynu (Baskin i Baskin, 2014) i dostępności pierwiastków, w tym metali i metali ciężkich na proces kiełkowania był szeroko badany (Kranner i Colville, 2011), nadal nie poznano wpływu czynników kontrastujących oba typy muraw (odczyn oraz dostępność żelaza, manganu i glinu) na zdolność nasion do wykiełkowania. Znacznie lepiej znane są przyczyny i skala problemu niedoborów mineralnych ograniczających wzrost kalcyfilów i kalcyfobów od momentu wschodu (Vélez-Bermúdez i Schmidt, 2022).

Uważa się, że głównym czynnikiem różnicującym biotop piaszczystych muraw bezwapiennych od kserotermicznych muraw wapieniolubnych jest wapń (Bothe, 2015), ale wśród pozostałych pierwiastków kontrastujących gleby kwaśne i zasadowe wymienić należy przede wszystkim żelazo, mangan i glin (Bothe, 2015).

Żelazo jest czwartym co do ilościowości pierwiastkiem w skorupie ziemskiej i jednocześnie najczęściej występującym w niej metalem ciężkim (Lautenschläger i wsp., 2007). Rozważając chemizm skał macierzystych, zawartość żelaza w piaskowcach jest przeszło dwa i pół razy wyższa niż w wapieniach (Turekian i Wedepohl, 1961). W glebach zawartość żelaza jest zmienna (0,02–5%) i związana jest z występowaniem licznych minerałów zawierających krzemiany, węglany, tlenki i wodorotlenki tegoż pierwiastka (Mocek, 2015). Żelazo występuje zarówno w fazie stałej, jak i w roztworze glebowym (w formie jonowej oraz skompleksowanej przez związki organiczne; Bothe, 2015; Mocek, 2015), a jego dostępność rośnie wraz ze spadkiem odczynu gleby. Żelazo jest niezbędne roślinom do przeprowadzania reakcji związanych z transportem elektronów, w tym fotosyntezy, oddychania i obrony antyoksydacyjnej; zaangażowane jest przez to w metabolizm pierwotny i specjalistyczny oraz wzrost i reakcje obronne (Rout i Sahoo, 2015).

Mangan jest czternastym co do ilościowości pierwiastkiem w skorupie ziemskiej i jednocześnie drugim najczęściej występującym w niej metalem ciężkim (Lautenschläger i wsp., 2007). Zawartość manganu w wapieniach jest ok. 10–100 razy większa niż w piaskowcach (Turekian i Wedepohl, 1961). Zawartość manganu w glebie waha się w granicach 0,2–1,0% i związana jest z obecnością jego tlenków, wodorotlenków i krzemianów (Mocek, 2015). Występuje zarówno w formie związanej w fazie stałej gleb, jak i w formie jonowej w roztworze glebowym, a jego dostępność (podobnie jak dostępność żelaza) rośnie wraz ze spadkiem odczynu gleby (Bothe, 2015; Mocek, 2015). Mangan jest mikroelementem pełniącym funkcje katalityczne w roślinach; zaangażowany jest w procesy fotosyntezy, oddychania i obrony antyoksydacyjnej, bierze przez to udział w reakcjach obronnych względem patogenów oraz w sygnalizacji komórkowej (Alejandro i wsp., 2020).

Glin jest trzecim najpowszechniejszym co do ilościowości pierwiastkiem występującym w skorupie ziemskiej i jednocześnie najczęściej występującym w niej metalem (Lautenschläger i wsp., 2007). Pod względem ilościowym glinu jest niemal sześć razy więcej w piaskowcach niż w wapieniach (Turekian i Wedepohl, 1961). Zawartość glinu w glebie jest bardzo zmienna (1–30%) i zależy od obecności minerałów zawierających krzemiany i wodorotlenki tego pierwiastka (Strawn i wsp., 2020). W glebie występuje zarówno w fazie stałej, jak i w roztworze glebowym, a jego dostępność rośnie wraz ze spadkiem odczynu gleby, przy czym specjacja tego pierwiastka w glebach jest skomplikowana (Bothe, 2015; Strawn i wsp., 2020). Glin nie jest uważany za pierwiastek niezbędny lub korzystny dla życia roślin i jego wpływ na rośliny dyskutowany jest najczęściej w kontekście toksyczności (Sade i wsp., 2016), choć ostatnio sugeruje się jego rolę w ograniczaniu objawów toksyczności zależnej od żelaza i manganu na glebach kwaśnych (Muhammad i wsp., 2019).

Niedobory żelaza są głównym źródłem zaburzeń dla wzrostu i funkcjonowania roślin występujących na glebach zasadowych (Lucena i Hernandez-Apaolaza, 2017). Obecnie uważa się, że zaburzenia te są wynikiem interakcji między złożoną matrycą gleby (w której wypadkowa procesów biogeochemicznych czyni żelazo niedostępnym) oraz rośliną wykazującą niski stopnień dopasowania do tejże gleby (tj. nie posiadającej cech gwarantujących utrzymanie homeostazy gospodarki żelazem; Colombo i wsp., 2014; Therby-Vale i wsp., 2022; Vélez-Bermúdez i Schmidt, 2022). Problem niedoborów żelaza można zatem rozpatrywać z perspektywy biotopu, jak i fitocenozy. Choć uważa się, że kalcyfile z definicji muszą być bardzo skuteczne w pobieraniu odpowiednich ilości żelaza z gleby zasadowej (Bothe, 2015), a kalcyfoby są lepiej dostosowane do toksyczności żelaza niż jego niedoboru (Bothe, 2015), to dowody na potwierdzenie tych zależności są słabe. Co więcej, część prac wskazuje, że częstość występowania ograniczeń zależnych od żelaza nie jest stała dla gatunku, tj. w zbliżonych warunkach siedliskowych osobniki wykazują różny stopień zaawansowania niedoboru (Grime and Hutchinson, 1967).

Wysycenie ziemskiej atmosfery tlenem spowodowało strącenie żelaza w formach nierozpuszczalnych, a to z kolei uniemożliwiło roślinom łatwy pobór tegoż pierwiastka i napędziło ewolucję strategii jego poboru (Vélez-Bermúdez i Schmidt, 2022). Obecnie u lądowych roślin wyższych obserwuje się dwie odmienne główne strategie poboru żelaza (*sensu* Römheld i Marschner, 1986): opartą na redukcji (Strategia I, występująca u roślin

innych niż trawy) oraz opartą na chelatacji (Strategia II, występująca u traw). Strategia I polega na następujących po sobie procesach lokalnego zakwaszenia ryzosfery przez zlokalizowane w błonie komórkowej H^+-ATPazy, redukcji jonu żelaza (z Fe^{3+} do Fe^{2+}) przez reduktazy oraz poboru dzięki odpowiednim przenośnikom (ang. *iron regulated transporter*, IRT; Chao i Chao, 2022). Strategia II związana jest z aktywnym wydzieleniem chelatorów (fitosideroforów), które kompleksują żelazo w formie utlenionej (Fe^{3+}) oraz poborem chelatu (chelatora będącego fitosideroforem i jonu) przez odpowiednie transportery (ang. *yellow stripe*, YS lub *yellow stripe-like*, YSL; Chao i Chao, 2022). Podział ten nie oddaje jednak w pełni rzeczywistych procesów zachodzących w ryzosferze, gdyż niektóre rośliny wykorzystują obie strategie jednocześnie lub wykazują przystosowania pośredniego typu (łączące procesy redukcji i chelatacji; Chao i Chao, 2022). Ze względu na powyższe, coraz większe problemy sprawia klasyfikacja i interpretowanie krzyżowego wykorzystania żelaza przez rośliny o różnych strategiach poboru tego pierwiastka (Dai i wsp., 2019) czy konkurencja o żelazo w formie chelatu między organizmami pochodzącymi z różnych domen (Winkelmann, 2007; Vélez-Bermúdez i Schmidt, 2022).

Niedobór żelaza może być powodowany zaburzeniami na wszystkich etapach jego transportu, a zatem podczas poboru z roztworu glebowego i/lub dystrybucji w organizmie, zarówno w wyniku zaburzeń transportu dalekiego na osi korzeń-pęd, jak i w wyniku problemów z transportem między apoplastem, a symplastem (Dey i wsp., 2020). Należy jednak zauważyć, że żadna z tych przyczyn nie pozwala na stworzenie ogólnego modelu tłumaczącego rozwój ograniczeń zależnych od żelaza, gdyż, przeciwnie do oczekiwań, często zdarza się, że rośliny wykazujące objawy chlorozy z powodu niedoboru żelaza zawierają znacznie więcej tegoż pierwiastka niż rośliny nie wykazujące objawów (Abadía i wsp., 2011). Przyczyniło się to utrwalenia pojęcia tzw. „paradoksu chlorozy zależnej od żelaza" (Abadía i wsp., 2011). Pomimo znaczącego przyrostu danych o strategiach poboru żelaza przez kalcyfile i kalcyfoby w przeciągu ostatnich kilkudziesięciu lat (Lee, 1999; Bothe, 2015; Vélez-Bermúdez i Schmidt, 2022), nie udało się określić w pełni powodów dla których rośliny te doświadczają ograniczeń związanych z żelazem.

Podstawowym, nierozstrzygniętym problemem pozostaje zatem rozłączność lub współwystępowanie określonych wymagań względem odczynu podłoża oraz dostępności żelaza. Nie jest również w pełni jasne, czy wymagania gatunków zasadolubnych (kalcyfilów) względem dostępności żelaza są zawsze niskie, a gatunków kwasolubnych (kalcyfobów) zawsze wysokie oraz jakie skutki pociągają za sobą niedobory tegoż pierwiastka. Dodatkowo sprawę tę, w przypadku gatunków Europy Środkowej i Wschodniej komplikuje fakt złożonej i nie do końca poznanej kwestii ewolucji kalcyfilności i kalcyfobowości (Chytrý i wsp., 2003; Tyler, 2003; Wolgemuth i Gigon, 2003). Warto również zaznaczyć, że różnice lub tożsamość tolerancji na badane czynniki na różnych stadiach ontogenezy nie zostały zbadane.

7. Uzasadnienie badań, cele pracy oraz ogólne hipotezy badawcze

Zarówno piaszczyste murawy bezwapienne, jak i kserotermiczne murawy wapieniolubne stanowią cenny obiekt badań geobotanicznych i doczekały się licznych opracowań florystycznych. Mimo to, tylko część roślin występujących w obrębie tychże muraw doczekało się szczegółowych badań w ujęciu ekologii gatunku. Stąd też niewiele wiadomo o ich amplitudzie ekologicznej, w tym o wymaganiach troficznych oraz zdolności do radzenia sobie z stresem warunkowanym czynnikami edaficznymi. Fragmentaryczność wiedzy pogłębia brak prac z zakresu ekologii porównawczej. Dlatego też stan zbadania tychże roślin należy określić jako niewystarczający.

Wśród przyczyn niskiego zainteresowania ekologią gatunków występujących w obrębie obu typów muraw należy wymienić utrudnienia wynikające z potrzeby poniesienia nakładów organizacyjnych dla prac z zakresu ekofizjologii i ekobiochemii, potrzeby interdyscyplinarnego podejścia do badań oraz stosunkowo niskiej świadomości względem wagi obu typów siedlisk w aspekcie poznawczym i aplikacyjnym. Ponadto, w kontekście biologii roślin, powyższy temat nie zyskał szerszej uwagi ze względu na tendencję do ekstrapolacji wyników badań nad roślinami modelowymi oraz uprawnymi na inne taksony, pomimo znacznych różnic strukturalno-funkcjonalnych między nimi, dzielącej ich odległości taksonomicznej, oraz różnic w ich niszach ekologicznych i pomiędzy zajmowanymi przez nie siedliskami. Dlatego też, niewiele wiadomo o wpływie chemicznych czynników edaficznych kontrastujących biotopy obu typów muraw (m.in. odczynu gleby oraz dostępności pierwiastków) na wzrost i funkcjonowanie roślin kwasolubnych i zasadolubnych. Ze względu na powyższe, zarówno ekofizjologiczne badania szczegółowe, jak i prace z zakresu ekologii porównawczej wydają się konieczne dla lepszego poznania i zrozumienia procesów kształtujących oba typy muraw.

W związku z powyższym, celem niniejszej pracy było:

- Zbadanie powiązania między indywidualnymi wymaganiami 12 gatunków roślin występujących w obrębie piaszczystych muraw bezwapiennych i kserotermicznych muraw wapieniolubnych względem odczynu gleby, a wymaganiami względem dostępności żelaza.
- Określenie częstości występowania chlorozy zależnej od żelaza dla badanych gatunków oraz złożoności przyczyn niedoborów tegoż pierwiastka.
- Zbadanie skutków fizjologicznych stresu powodowanego niedopasowaniem względem przeciwstawnego (względem optymalnego) typu gleby.
- Określenie wpływu chemicznych czynników edaficznych kontrastujących oba typy muraw na zdolność do wykiełkowania (zakończenia procesu kiełkowania) nasion 20 gatunków roślin z piaszczystych muraw bezwapiennych i kserotermicznych muraw wapieniolubnych.

Osiągnięcie powyższych celów realizowane było w ścisłym połączeniu z testowaniem odpowiadających im hipotez ogólnych:

- W obrębie obu badanych typów muraw występują gatunki o preferencjach substratowych zgodnych z ogólną wiedzą o badanych siedliskach, tj. gatunki kwasolubne (kalcyfoby) występujące w obrębie piaszczystych muraw bezwapiennych preferują glebę kwaśną, a gatunki zasadolubne (kalcyfile) występujące w obrębie kserotermicznych muraw wapieniolubnych preferują glebę zasadową, oraz ich wymagania względem dostępności żelaza są zgodne z dostępnością tegoż pierwiastka w glebie optymalnej dla wzrostu, tj. gatunki kwasolubne wymagają więcej żelaza niż gatunki zasadolubne (pierwsza hipoteza ogólna; HO–1).
  Testowanie pierwszej hipotezy ogólnej realizowane było poprzez testowanie hipotez szczegółowych z prac P–1, P–2 i P–3 oraz w oparciu o podsumowującą analizę zbiorczą wyników przedstawionych w tychże pracach.
- Gatunki kwasolubne (niedopasowane do gleb o odczynie zasadowym) są bardziej podatne na rozwój chlorozy zależnej od niedoboru żelaza na glebie zasadowej niż gatunki zasadolubne (dopasowane do gleb o odczynie zasadowym), a dysfunkcja ta powodowana jest więcej niż jednym czynnikiem, co oznacza, że etiologia niedoboru ma charakter złożony (druga hipoteza ogólna; HO–2).
  Testowanie drugiej hipotezy ogólnej realizowane było poprzez testowanie hipotez szczegółowych z prac P–1, P–2 i P–3 oraz w oparciu o podsumowującą analizę zbiorczą wyników przedstawionych w tychże pracach.
- Stres powodowany skrajnym niedopasowaniem względem gleby (podczas wzrostu na przeciwstawnym typie gleby) występuje zarówno u gatunków kwasolubnych, jak i zasadolubnych, a stres powodowany niedoborem żelaza ma uniwersalny charakter na poziomie aparatu fotosyntetycznego i związany jest również ze zmianami aktywności enzymatycznego systemu antyoksydacyjnego (gatunki kwasolubne) lub metabolizmem specjalistycznym (gatunki zasadolubne) badanych gatunków (trzecia hipoteza ogólna; HO–3).
  Testowanie trzeciej hipotezy ogólnej realizowane było poprzez testowanie hipotez szczegółowych z prac P–1, P–2 i P–3.
- Tolerancja badanych gatunków względem dostępności żelaza, manganu, glinu oraz odczynu, stanowiąca element strategii kiełkowania badanych gatunków, jest zgodna z warunkami edaficznymi spodziewanymi w obrębie badanych muraw, tj. gatunki kwasolubne będą lepiej tolerowały wyższe stężenia żelaza, manganu i glinu niż gatunki zasadolubne, przy czym jednocześnie gatunki kwasolubne będą bardziej tolerancyjne względem kwaśnego odczynu medium oraz żelaza i manganu w formie jonowej, a gatunki zasadolubne będą bardziej tolerancyjne względem zasadowego odczynu medium oraz żelaza i manganu w formie chelatów. Dodatkowo, preferencje te będą związane z wartością liczby wskaźnikowej Ellenberga dla odczynu (EIV R) i/lub masą diaspor (czwarta hipoteza ogólna; HO–4).
  Testowanie czwartej hipotezy ogólnej realizowane było poprzez testowanie hipotez szczegółowych z pracy P–4.

8. Materiał i metody

a. Materiał wykorzystany w trakcie realizacji badań

Do badań nad reakcjami roślin na rodzaj gleby i dostępność żelaza w glebie zasadowej wykorzystano dwa reprezentatywne typy gleb: kwaśną glebę bielicową i zasadową rędzinę właściwą. Parametry fizykochemiczne (Tabela 1) oraz metodykę ich oznaczeń podano w pracy P–1. Oba typy gleb wykorzystano w pracach P–1, P–2 oraz P–3.

Tabela 1. Właściwości fizykochemiczne gleb wykorzystywanych w badaniach.

Typ[1]	Gleba bielicowa (Entic Podzol)	Rędzina właściwa (Rendzic Leptosol)
Gatunek[2]	Piasek luźny (sand)	Glina piaszczysta (sandy loam)
Kwasowość wymienna (pH_{KCl})	4.3 ± 0.2	7.3 ± 0.3
Zawartość węgla organicznego (C_{org}) [%]	0.23 ± 0.05	2.66 ± 0.48
Całkowita zawartość azotu (N_{total}) [%]	0.021 ± 0.003	0.220 ± 0.031
Zawartość dostępnego fosforu (P) [mg kg^{-1} gleby]	56.0 ± 8.0	46.0 ± 7.0
Zawartość dostępnego potasu (K) [mg kg^{-1} gleby]	<10.0*	246.0 ± 36.0
Zawartość dostępnego magnezu (Mg) [mg kg^{-1} gleby]	37.0 ± 6.0	94.0 ± 15.0
Zawartość dostępnego manganu (Mn) [mg kg^{-1} gleby]	<10.0*	88.1 ± 13.2
Zawartość dostępnej miedzi (Cu) [mg kg^{-1} gleby]	nb.	1.8 ± 0.3
Zawartość dostępnego cynku (Zn) [mg kg^{-1} gleby]	nb.	<2.5*
Zawartość dostępnego żelaza (Fe) [mg kg^{-1} gleby]	503 ± 86	414 ± 70
Zawartość dostępnego wapnia (Ca) [mg kg^{-1} gleby]	<40*	3415 ± 649
Średni masowy stosunek C:N	10.95	12.01
Średni masowy stosunek N:P	0.38	4.78
Wykorzystano w pracy:	P–1, P–2, P–3	P–1, P–2, P–3

[1]polska nazwa typu podana wg. Systematyki Gleb Polski (Marcinek i Komisarek, 2011) jako najbardziej bliskoznaczny odpowiednik nazwy angielskiej; angielska nazwa podana według Światowej Bazy Referencyjnej Zasobów Glebowych (ang. *World Reference Base for Soil Resources*; IUSS Working Group, 2015). [2]polska nazwa gatunku podana wg. Systematyki Gleb Polski (Marcinek i Komisarek, 2011) jako najbardziej bliskoznaczny odpowiednik nazwy angielskiej; angielska nazwa podana według Departamentu Rolnictwa Stanów Zjednoczonych (ang. *United States Department of Agriculture*; Ditzler I wsp., 2017). nb. – nieobecny. * – obecny, poniżej podanego progu oznaczalności.

Do badań wykorzystano łącznie 24 gatunki występujące naturalnie w obrębie badanych typów muraw: *Alyssum montanum* L., *Antennaria dioica* (L.) Gaertn., *Aster amellus* L., *Betonica officinalis* L. (syn. *Stachys officinalis* (L.) Trevis.), *Centaurea scabiosa* L., *Centaurea stoebe* Tausch, *Dianthus carthusianorum* L., *Dianthus deltoides* L., *Echium vulgare* L., *Gentiana cruciata* L., *Hieracium pilosella* L. (*sensu lato*), *Hypericum perforatum* L., *Hypochaeris radicata* L., *Jasione montana* L., *Plantago media* L., *Potentilla arenaria* Borkh. ex G.Gaertn., B.Mey. & Scherb., *Potentilla recta* L., *Prunella grandiflora* (L.) Scholler, *Rumex acetosella* L., *Salvia verticillata* L., *Stachys germanica* L., *Thymus serpyllum* L., *Verbascum thapsus* L., *Veronica teucrium* L. (Tabela 2). Pod względem taksonomicznym, badane gatunki należą do 21 rodzajów przynależących do 12 rodzin. Stosunkowo duża liczba gatunków miała na celu wspomóc badanie zróżnicowania reakcji roślin z danej grupy ekologicznej względem typu gleby i dostępności żelaza (prace P–1, P–2 i P–3; 12 gatunków

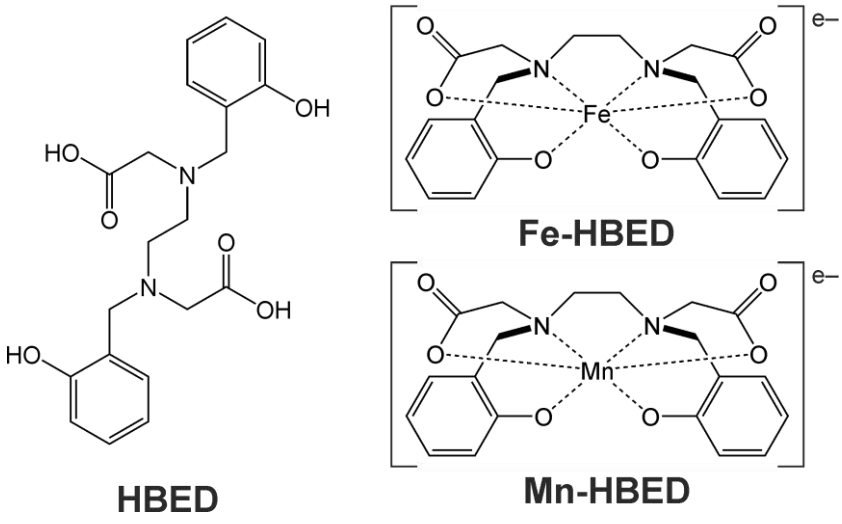
łącznie) oraz wymagań względem odczynu i dostępności żelaza, manganu i glinu w procesie kiełkowania (praca P–4; 20 gatunków łącznie). Ponadto, szerokie zróżnicowanie taksonomiczne gatunków miało na celu redukcję błędu związanego z ekstrapolacją wniosków wyciągniętych z badań nad ekologią ograniczonej liczby gatunków na ekologię zbiorowisk. Dodatkowymi kryteriami wyboru było również: występowanie w obrębie piaszczystych muraw bezwapiennych (P–2, P–3 i P–4) lub kserotermicznych muraw wapieniolubnych (P–1, P–3 i P–4), wieloletni lub przynajmniej dwuletni typ wzrostu (P–1, P–2, P–3 i P–4), łatwość uzyskania odpowiedniej biomasy osobników (P–1, P–2 i P–3), łatwość w pozyskaniu dużej ilości diaspor z jednego sezonu wegetacyjnego (P–4) oraz przynależność do kladu dwuliściennych właściwych (trójbruzdowe; *Eudicotyledoneae* Donoghue, Doyle et Cantino), a przez to ogólne podobieństwo strategii poboru żelaza – Strategia I (P–1, P–2, P–3 i P–4). Do badań nie włączano gatunków z rodziny Fabaceae Lindl., które nawiązują symbiozę z bakteriami wiążącymi azot atmosferyczny (ze względu na duże zapotrzebowanie tychże mikroorganizmów na żelazo; Brear i wsp., 2013).

Tabela 2. Spis gatunków wykorzystanych w badaniach, ich przynależność taksonomiczna oraz wstępnie zakładana preferencja względem odczynu gleby.

Nazwa gatunkowa	Rodzina	Zakładana preferencja względem odczynu gleb[1]	Wykorzystano w pracy:			
			P–1	P–2	P–3	P–4
Alyssum montanum	Brassicaceae	A		•		•
Antennaria dioica	Asteraceae	A		•		
Aster amellus	Asteraceae	B	•			•
Betonica officinalis	Lamiaceae	B	•			•
Centaurea scabiosa	Asteraceae	B			•	•
Centaurea stoebe	Asteraceae	A			•	•
Dianthus carthusianorum	Caryophyllaceae	B				•
Dianthus deltoides	Caryophyllaceae	A				•
Echium vulgare	Boraginaceae	A				•
Gentiana cruciata	Gentianaceae	B				•
Hieracium pilosella	Asteraceae	A				•
Hypericum perforatum	Hypericaceae	A				•
Hypochaeris radicata	Asteraceae	A		•		•
Jasione montana	Campanulaceae	A		•		
Plantago media	Plantaginaceae	B		•		
Potentilla arenaria	Rosaceae	A				•
Potentilla recta	Rosaceae	B				•
Prunella grandiflora	Lamiaceae	B	•			•
Rumex acetosella	Polygonaceae	A				•
Salvia verticillata	Lamiaceae	B	•			
Stachys germanica	Lamiaceae	B				•
Thymus serpyllum	Lamiaceae	A				•
Verbascum thapsus	Scrophulariaceae	A				•
Veronica teucrium	Plantaginaceae	B	•			•
		Suma:	5	5	2	20

[1]A – gatunek kwasolubny występujący głównie w obrębie piaszczystych muraw bezwapiennych (kalcyfob); B – gatunek zasadolubny występujący głównie w obrębie kserotermicznych muraw wapieniolubnych (kalcyfil).

Badane układy doświadczalne (według opisów podanych w pracach) zakładały pobudzenie poboru żelaza przez rośliny w wyniku zwiększenia zawartości i dostępności tegoż pierwiastka w glebie. Do badań wybrano żelazo w formie chelatu – pozwoliło to na jednoczesne uniknięcie problemu strącania form nierozpuszczalnych żelaza z formy jonowej (Colombo i wsp., 2014) oraz konieczności interpretacji wyników w kontekście zakwaszenia ryzosfery przez badane rośliny (w celu zwiększenia dostępności pierwiastka). Związkiem kompleksującym (chelatorem) był kwas *N,N'*-di(2-hydroksybenzylo)-etylenodiamino-*N,N'*-dioctowy (HBED; wzór sumaryczny $C_{20}H_{24}N_2O_6$; Rycina 1) należący do grupy syntetycznych ligandów acyklicznych (Wahsner i wsp., 2019). HBED wybrano ze względu na wysoką trwałość w glebie (Bin i wsp., 2016), wysoką specyficzność względem żelaza w porównaniu do innych pierwiastków (umożliwiającą założenie, że po pobraniu żelaza z chelatu przez roślinę, chelator ten będzie dążył do dalszego kompleksowania żelaza; L'Eplattenier i wsp., 1967; Norvell, 1991) oraz wysoką stabilność chelatacji w szerokim zakresie wartości pH (umożliwiającą odtworzenie warunków wysoce zbliżonych do naturalnych; Norvell, 1991; López-Rayo i wsp., 2009). Ponadto, żelazo w Fe-HBED występuje na trzecim stopniu utlenienia, co oznacza, że musi ono przejść przez kluczowy etap redukcji enzymatycznej w trakcie poboru przez roślinę. Przybliża to układ doświadczalny do warunków naturalnych. Fe-HBED wykorzystano w pracach P–1, P–2, P–3 i P–4. Wybór Mn-HBED jako chelatu do badań nad strategiami kiełkowania podyktowany był koniecznością zapewnienia w pełni czynnikowego układu doświadczalnego. Chelat ten wykorzystano w pracy P–4. Wybór chelatów do badań nad strategiami kiełkowania podyktowany był brakiem jakichkolwiek informacji literaturowych odnoszących się do wpływu chelatów żelaza i manganu na proces kiełkowania. Ponadto, Mn-HBED nie był badany wcześniej w kontekście wpływu na organizmy.



Rycina 1. Wzory chemiczne HBED oraz Fe-HBED i Mn-HBED. Strukturę chemiczną HBED podano za bazą PubChem (National Center for Biotechnology Information, 2023). Strukturę chemiczną Fe-HBED oraz Mn-HBED podano według literatury (Wahsner i wsp., 2019); wartość *e* wynosi: w cząsteczce Fe-HBED: *e* = 1 dla Fe(III), w cząsteczce Mn-HBED: *e* = 1 dla Mn(III) lub *e* = 2 dla Mn(II) (Pinto i wsp., 2019; Wahsner i wsp., 2019).

b. Metody doświadczalne wykorzystane w trakcie realizacji badań

W pracy wykorzystano dwie metody doświadczalne:

- **Polowe doświadczenie doniczkowe** – częściowo kontrolowane doświadczenie pozwala na prowadzenie badań nad roślinami w warunkach czasowej zmienności warunków zewnętrznych przy znacznym ograniczeniu zmienności przestrzennej tychże warunków (Poorter, 2012). Rośliny wzrastają zatem w warunkach zbliżonych do środowiska naturalnego. Zaletą tego podejścia jest ujednolicenie początkowych warunków doświadczenia i ich przestrzennej zmienności (ta sama objętość i jednorodność substratu, jakość i ilość światła, temperatura, dostępność wody, skład atmosfery i czynniki regulujące ewapotranspirację), co przekłada się na większą homogenność uzyskanych wyników niż dla doświadczeń prowadzonych w gruncie. Tym samym, zapewniona zostaje powtarzalność i odtwarzalność badania, przy czym oddziaływanie środowiska zewnętrznego stwarza możliwość interpretacji wyników w kontekście środowiska naturalnego. Wadą doświadczeń polowych jest nieprzewidywalna zmienność czasowa głównych czynników środowiska, tj. temperatury, oświetlenia i składu atmosfery. Metodę wykorzystano w pracach P–1, P–2 oraz P–3.
- **Laboratoryjne doświadczenie szalkowe** – ściśle kontrolowanie doświadczenie laboratoryjne z wykorzystaniem szalek Petriego i regulowanej inkubacji pozwala na pełną kontrolę czynników (stabilizację wszystkich przewidywalnych punktów swobody), a przez to sprawdzenie zdolności nasion do wykiełkowania (zakończenia procesu kiełkowania; Baskin i Baskin, 2014). W takim przypadku, kontroli podlegają wszystkie główne czynniki wpływające na kiełkowanie: fototermoperiod, wilgotność, parametry podłoża i medium oraz natężenie czynników badanych. Dzięki powyższemu, można uniknąć interferencji z niekontrolowanym działaniem czynników zewnętrznych. Kontrolując rodzaj podłoża oraz medium w którym zachodzi proces kiełkowania (w tym przypadku odpowiednio niskopopiołowe sączki analityczne oraz woda najwyższej klasy czystości) unika się problemu wpływu złożonej matrycy (gleby) na proces biologiczny. To z kolei, przy stabilizacji wszystkich (poza czynnikiem badanym) przewidywalnych punktów swobody (w położeniu optimum), stwarza możliwość interpretacji wyników w kontekście czynnika zaburzającego. Natomiast w przypadku braku indukowania zaburzeń, tj. stabilizacji wszystkich przewidywalnych punktów swobody (w położeniu optimum), wyniki można interpretować w kontekście stanu fizjologicznego nasion. Ponadto, wyrównanie doświadczenia pozwala na porównawczą interpretację zjawisk w kontekście czasowym. Słabą stroną tego typu badań jest brak odwzorowania wpływu złożonej matrycy środowiskowej na proces kiełkowania oraz kosztochłonność, pracochłonność i potrzeba stworzenia zaplecza badawczego. Podobnie jak inne typy doświadczeń nad zdolnością nasion do wykiełkowania, układ ten uniemożliwia monitoring parametrów chemicznych medium w trakcie trwania procesu (brak możliwości ilościowego badania chemicznego bez zaburzenia układu). Metodę wykorzystano w pracy P–4.

c. Techniki wykorzystane w trakcie realizacji badań

W pracy wykorzystano pięć technik analitycznych do analizy materiału roślinnego:

- **Metody wagowe** – techniki ilościowego określenia masy organów roślin oraz stosunków masowych (Pérez-Harguindeguy i wsp., 2013). W połączeniu z suszeniem powietrznym lub wspomaganym termicznie pozwalają na określenie odpowiednio powietrznie suchej masy lub suchej masy próby badanej. Technika została wykorzystana do określenia świeżej oraz suchej masy organów badanych roślin (prace P–1, P–2 i P–3) oraz powietrznie suchej masy diaspor (prace P–1, P–2 i P–4). Pomiary przeprowadzono przy pomocy wagi precyzyjnej PS 210.R2 oraz wagi analitycznej AS 62.R2 produkcji Radawag (Polska), w zależności od potrzeb analitycznych.
- **Laserowy skaning optyczny** – technika ilościowego określania powierzchni obiektów, w tym dwuwymiarowych. Oparta jest o wysokorozdzielczą rejestrację intensywności odbitego światła laserowego. Metoda oparta o wykorzystanie światła czerwonego (λ = 675 nm) często znajduje zastosowanie w jedno- i dwuwymiarowej analizie obiektów (Bourouina i wsp., 2005). Technika została wykorzystana do określenia wielkości liści (prace P–1, P–2 i P–3), tj. pomiaru ich powierzchni, obwodu, długości oraz szerokości. Pomiary przeprowadzono przy pomocy przenośnego skanera laserowego CI 202 produkcji CID Bio-Science (Stany Zjednoczone).
- **Spektrofluorymetria** – technika z zakresu spektroskopii molekularnej pozwalająca na ilościowy pomiar intensywności światła oddanego z próby w procesie zaniku promienistego w wyniku przejścia elektronów ze stanu wzbudzenia do stanu podstawowego (Szczepaniak, 2012). Metoda spektrofluorymetryczna oparta o rejestrację intensywności fluorescencji chlorofilu mierzonej przy dwóch długościach fali emisji (λ_1 = 700 nm i λ_2 = 735 nm) pozwala na ilościowy pomiar zawartości chlorofilu w przeliczeniu na jednostkę powierzchni. Technika ta została wykorzystana do analizy zawartości chlorofilu w liściach (prace P–1, P–2 i P–3). Pomiary przeprowadzono przy pomocy przenośnego spektrofluorymetru CCM300 produkcji Opti-Sciences (Stany Zjednoczone). Metoda spektrofluorymetryczna oparta o rejestrację szybkich zmian w intensywności fluorescencji chlorofilu *a* po podaniu pojedynczego impulsu światła wysycającego na liście adaptowane w ciemności (tzw. test OJIP) pozwala na ilościowy pomiar funkcjonowania fotoukładu II (PSII) oraz, pośrednio, informuje o warunkach w których zachodzi fotosynteza. Technika ta została wykorzystana do analizy fluorescencji chlorofilu *a* w liściach (prace P–1, P–2 i P–3). Pomiary przeprowadzono przy pomocy przenośnego spektrofluorymetru Fluorpen FP100 produkcji Photon Systems Instruments (Republika Czeska).
- **Spektrofotometria** – technika z zakresu spektroskopii molekularnej pozwalająca na badania widm elektronowych oraz na ilościowy pomiar intensywności światła zaabsorbowanego przez próbę (Szczepaniak, 2012). Spektrofotometria UV-VIS pozwala na ilościowy pomiar stężenia analitów dzięki wprost proporcjonalnej relacji między absorbancją jednorodnego roztworu, a jego stężeniem i grubością warstwy

absorbującej światło monochromatyczne w zakresie ultrafioletu (UV) lub światła widzialnego (VIS). Technika ta została wykorzystana do ilościowej analizy aktywności enzymów: dysmutazy ponadtlenkowej, katalazy i peroksydazy (P–1), zawartości białka (P–1) oraz zawartości metabolitów specjalistycznych: związków fenolowych, fenylopropanoidów, *orto*-dihydroksyfenoli, flawonoidów i flawan-3-oli (P–2) i zdolności antyoksydacyjnej metodą FRAP (P–2), a także pomocniczo, do analizy spektralnej Fe-HBED i Mn-HBED (P–4). Pomiary przeprowadzono przy pomocy spektrofotometru UV-VIS Unicam UV 300 produkcji Unicam (Wielka Brytania; P–1) oraz czytnika wielodetekcyjnego SpectraMax i3 produkcji Molecular Devices (Stany Zjednoczone; P–2).

- **Absorpcyjna spektrometria atomowa** – technika z zakresu spektrometrii atomowej pozwalająca ilościowy pomiar stężenia pierwiastków w próbie w oparciu o zdolność swobodnych atomów do absorpcji promieniowania elektromagnetycznego w warunkach plazmy niskotemperaturowej (Szczepaniak, 2012). Zarówno płomieniowa absorpcyjna spektrometria atomowa (ang. *flame atomic absorption spetrometry*, F-AAS), jak i elektrotermiczna absorpcyjna spektrometria atomowa (ang. *electrothermal atomic absorption spetrometry*, ET-AAS) pozwalają na określenie zawartości pierwiastków w złożonych matrycach roślinnych. Technika ta została wykorzystana do ilościowej analizy zawartości wapnia, magnezu, żelaza, manganu, cynku i miedzi w organach badanych gatunków (prace P–1, P–2 i P–3). Pomiary przeprowadzono przy pomocy spektrometru absorpcji atomowej Varian SpectrAA 300 produkcji Varian (Australia).

Dodatkowo, w pracy wykorzystano pomocniczo trzy techniki analityczne do badań właściwości chelatów oraz badanych roztworów:

- **pH-metria** – technika z zakresu potencjometrii pozwalająca na ilościowy pomiar aktywności jonów wodorowych w roztworze poprzez oznaczenie siły elektromotorycznej ogniwa złożonego z elektrody wskaźnikowej i porównawczej zanurzonej w roztworze (Szczepaniak, 2012). Technika ta została wykorzystana pomocniczo do ilościowej analizy odczynu buforów fosforanowych oraz roztworów soli glinu oraz roztworów soli i chelatów manganu i żelaza (praca P–4).
- **Absorpcyjna spektroskopia podczerwieni z transformacją Fouriera (FT-IR)** – technika z zakresu spektroskopii pozwalająca na jakościową i ilościową analizę prób poprzez analizę wyekstrahowanego dzięki transofrmacji Fouriera widma interferencyjnego próby (Smith, 2011). Technika ta została wykorzystana pomocniczo do analizy chelatów Fe-HBED i Mn-HBED (praca P–4).
- **Chromatografia cieczowa z tandemową spektrometrią mas (LC-MS/MS)** – technika z zakresu chromatografii pozwalająca na rozdzielanie mieszanin dzięki podziałowi składników mieszanin między fazę stacjonarną i mobilną układu chromatograficznego oraz na ilościową i jakościową analizę spektrometryczną tychże składników po rozdziale na podstawie stosunku masy do ładunku ich naładowanych cząsteczek (Szczepaniak, 2012). Technika ta została wykorzystana pomocniczo do analizy chelatów Fe-HBED i Mn-HBED (praca P–4).

Szczegółowe informacje odnoszące się procedur przeprowadzonych analiz, wraz z odniesieniami literaturowymi do metodyki, znajdują się w pracach oryginalnych, zgodnie z podanymi powyżej informacjami.

d. Oprogramowanie wykorzystane w trakcie realizacji badań

W pracy wykorzystano następujące oprogramowanie:

- **Statistica** – oprogramowanie pozwalające na analizę statystyczną oraz wizualizację danych. Oprogramowanie (wersja 12.0 dla P–1 oraz wersja 13.3 dla P–2, P–3 i P–4 oraz dla obliczeń uzupełniających) wykorzystano do: analizy normalności rozkładu testem Kołmogorowa-Smirnowa; analizy homogeniczności wariancji testem Browna-Forsythea; jedno-, dwu- i trzyczynnikowej analizy wariancji (ang. *analysis of variance*, ANOVA); analizy *post hoc* testem rozsądnej istotnej różnicy Tukeya; analizy *post hoc* testem Bonferroniego; analizy korelacji liniowej Pearsona; analizy korelacji rang Spearmana oraz analizy składowych głównych (ang. *principal component analysis*, PCA) opartej o matrycę korelacji.
- **R** – platforma programistyczna oparta o język programowania R, pozwalająca, przy wykorzystaniu odpowiednich zbiorów bibliotek (pakietów), na analizę statystyczną oraz wizualizację danych (R Core Team, 2023). Oprogramowanie (wersja 3.5.2) wraz z pakietem `germinationmetrics` (wersja 0.1.3; Aravind i wsp., 2020) wykorzystano w pracy P–4 do obliczeń wskaźnika szybkości kiełkowania (ang. *index of germination velocity*, IGV)
- **Clustvis** – oprogramowanie on-line oparte o język programowania R wykorzystujące zróżnicowane pakiety analizy statystycznej oraz wizualizacji danych (Metsalu i Vilo, 2015), pozwalające na przeprowadzenie hierarchicznej analizy skupień (ang. *hierarchical cluster analysis*, HCA) oraz analizy składowych głównych (ang. *principal component analysis*, PCA). Oprogramowanie wykorzystano w pracy P–4 do hierarchicznej analizy skupień (HCA).

Szczegółowe informacje odnoszące się procedur statystycznych oraz użycia programów Statistica, R oraz ClustVis znajdują się w pracach oryginalnych, zgodnie z podanymi powyżej informacjami.

Oznaczenia spektrofotometryczne, spektrofluorymetryczna oraz oparte o absorpcyjną spektrometrię atomową prowadzono z wykorzystaniem oprogramowania dedykowanego urządzeniom: Vision32 dla spektrofotometru Unicam UV 300, SoftMax Pro dla czytnika wielodetekcyjnego SpectraMax i3, Fluorpen dla spektrofluorymetru Fluorpen FP100 oraz SpectrAA dla spektrometru absorpcji atomowej SpectrAA 300.

9. Szczegółowe omówienie wyników

Praca P–1

W ramach pierwszego etapu prac zweryfikowano hipotezy związane z podatnością roślin wybitnie wapieniolubnych na rozwój ograniczeń żywieniowych zależnych od żelaza. Badanie miało na celu określenie zróżnicowania indywidualnych wymagań badanych gatunków względem dostępności żelaza, poznanie etiologii niedoborów tego pierwiastka i jego funkcjonalnych skutków. Do doświadczeń wybrano pięć gatunków roślin (*A. amellus*, *B. officinalis*, *P. grandiflora*, *S. verticillata* i *V. teucrium*) występujących naturalnie na glebach o zasadowym odczynie w obrębie kserotermicznych muraw wapieniolubnych.

W celu przetestowania pierwotnych założeń, zaprojektowano porównawczy układ doświadczalny, w którym rośliny wzrastały na czterech typach podłoża: 1) kwaśnej glebie bezwapiennej (gleba bielicowa; wariant „p"), 2) zasadowej glebie wapiennej (rędzinie właściwej; wariant „r"), zasadowej glebie wapiennej zasilanej suboptymalną dawką żelaza w formie chelatu (5 µmol · kg^{-1} gleby; wariant „r5") oraz zasadowej glebie wapiennej zasilanej optymalną dawką żelaza w formie chelatu (25 µmol · kg^{-1} gleby; wariant „r25"). Oba wykorzystane typy gleb pochodziły ze stanowisk półnaturalnych. Pod względem parametrów fizyko-chemicznych, zarówno gleba bielicowa, jak i rędzina właściwa przejawiały cechy typowe dla ubogich gleb mineralnych. Fe-HBED został wybrany jako źródło Fe ze względu na wysoką stabilność oraz dostępność dla roślin w warunkach alkalicznych, co odzwierciedla proces chelatacji tego pierwiastka na glebach o odczynie zasadowym. Dawki Fe-HBED dobrano w oparciu o dane odnoszące się do przeciętnej dostępności tego pierwiastka zapewniającej optymalny rozwój znacznej części lądowych roślin wyższych (Asher i Edwards, 1983) oraz w oparciu o prace podejmujące problem wymagań roślin kwasolubnych i zasadolubnych względem dostępności żelaza (Venturas i wsp., 2014).

Powyższy układ doświadczalny pozwalał w swych założeniach na jednoczesną falsyfikację następujących hipotez badawczych: hipoteza pierwsza: badane gatunki mają wyraźne preferencje względem typu gleby i są gatunkami wapieniolubnymi (rosną lepiej na zasadowej rędzinie właściwej niż na kwaśnej glebie bielicowej; porównanie wariantów „p" i „r"; H1); hipoteza druga: wszystkie badane gatunki są w stanie zaspokoić swoje potrzeby żywieniowe gdy rosną na glebie o odczynie zasadowym (tj. rosną na badanej glebie nie wykazując objawów niedoborów składników odżywczych; brak różnic fenotypowych w obrębie wszystkich testowanych wariantów; H2); hipoteza trzecia: jeśli któryś z badanych gatunków wykazuje niedobory składników mineralnych, to są to niedobory żelaza (tj. w przypadku wystąpienia chloroz na glebie alkalicznej, objaw ten może zostać zniesiony poprzez doglebowe podanie żelaza w formie chelatu; zawartość chlorofilu jest istotnie statystycznie większa w wariancie „r5" i/lub „r25" niż w wariancie „r"; H3); hipoteza czwarta: obserwowane niedobory żelaza są w istocie ograniczeniami o złożonej etiologii (tj. zarówno ich wystąpienie, jak i zniesienie współwystępuje ze zmianami w poborze i/lub alokacji pierwiastków innych niż żelazo; zawartość jednego lub kilku pierwiastków

lub ich stosunek ilościowy do żelaza będzie ulegał istotnej statystycznie zmianie wraz ze wzrostem dawki chelatu w sekwencji „r", „r5" i „r25"; H4).

Doświadczenie potwierdziło, że większość badanych gatunków wykazuje silne wymagania względem zasadowego odczynu gleby – *A. amellus*, *P. vulgaris* oraz *S. verticillata* rosły istotnie lepiej na rędzinie właściwej niż na glebie bielicowej, podczas gdy *B. officinalis* oraz *V. teucrium* nie przejawiały wyraźnych preferencji względem badanych typów gleb. Tym samym, wykazując zróżnicowanie wymagań badanych gatunków względem warunków edaficznych, odrzucono roboczą hipotezę pierwszą (H1) o jednorodności preferencji glebowych w obrębie kserotermicznych muraw wapieniolubnych. Sugeruje to, że podczas gdy część roślin zasiedlających badany typ siedliska posiada wąski zakres tolerancji względem odczynu gleby (gatunki stenotopowe), pozostała ich część jest w stanie zajmować bardziej zróżnicowane typy siedlisk (gatunki eurytopowe).

Analiza zawartości chlorofilu w liściach oraz klasyfikacja intensywności chlorozy w oparciu o skalę fenotypową pokazały wyraźnie, że *A. amellus*, *B. officinalis* oraz *P. grandiflora* nie są w stanie zaspokoić swoich potrzeb żywieniowych na glebach zasadowych w warunkach uprawy izolowanej – rośliny rosnące na rędzinie właściwej wykazywały objawy chlorozy międzynerwowej (o intensywności zależnej od gatunku) typowej dla niedoborów żelaza. Powyższe dane przyczyniły się do odrzucenia hipotezy drugiej (H2). Z drugiej strony, nie zaobserwowano istotnych ilościowych i/lub jakościowych zmian dla *S. verticillata* i *V. teucrium* odnoszących się do pigmentacji blaszek liściowych w tożsamych warunkach uprawy. Wskazuje to zróżnicowanie indywidulanych wymagań względem dostępności pierwiastków zaangażowanych w biosyntezę i/lub utrzymanie chlorofilu. Jednocześnie, wymagania względem pierwiastków zapewniających ciągłość utrzymania funkcji chloroplastów nie są tożsame z warunkami zapewniającymi optymalny wzrost wegetatywny. Ponadto, rozwój objawów niedoborów mineralnych w uprawie kontrolowanej na glebie zasadowej (silnie zbliżonej do optymalnej siedliskowo) sugeruje przyczyny zależne od czynników, które nie były analizowane i/lub odtwarzane z natury (w tym przede wszystkim zależności wewnątrz- i międzygatunkowe).

Doświadczalne traktowanie roślin Fe-HBED znosiło objawy chlorozy na glebie zasadowej, a dawka chelatu przełamująca objawy niedoboru żelaza była zależna od gatunku. Efekt łagodzący stres wywołany niedoborami mineralnymi obserwowano również w kontekście pozostałych zbadanych aspektów funkcjonalnych, w tym na poziomie fotoukładu II (badanie fluorescencji chlorofilu *a*) oraz z perspektywy aktywności enzymów antyoksydacyjnych (dysmutazy ponadtlenkowej, katalazy oraz peroksydazy). Ze względu na wysoką selektywność w stosunku do żelaza oraz wysoką stałą trwałości kompleksu Fe-HBED (L'Eplattenier i wsp., 1967), stwierdzono, że badane objawy niedoborów mineralnych są ograniczeniami zależnymi od żelaza (potwierdzono hipotezę trzecią; H3). Jednakże, badanie składu mineralnego nie pozwoliło na jednoznaczne powiązanie redukcji nasilenia chlorozy z zwiększeniem zawartości żelaza w częściach nadziemnych roślin lub zmianami w translokacji tegoż pierwiastka na osi korzeń–pęd. Notowano przy tym jednocześnie wyraźny spadek zawartości manganu wraz ze wzrostem dostępności żelaza

w formie chelatu, co silnie wskazuje na udział manganu w powstawaniu ograniczeń zależnych od żelaza. Wyniki wskazują, że nie ma podstaw do odrzucenia hipotezy czwartej (H4), co sugeruje złożoną etiologię obserwowanego zjawiska. Obserwacja ta skłania do rozważenia relacji między zaburzeniem ilościowego stosunku dostępności żelaza i manganu, a niską specyficznością transporterów odpowiedzialnych za pobór obu pierwiastków z gleby w kształtowaniu chlorozy zależnej od żelaza. Rozwiązanie to wpisuje się w aktualne spojrzenie na chlorozę zależną od żelaza (Therby-Vale i wsp., 2022) i przyczynia częściowo do wyjaśnienia tzw. „paradoksu chlorozy zależnej od żelaza" dotykającego roślin występujących w obrębie kserotermicznych murawy wapieniolubnych, wskazując, że etiologia tegoż ograniczenia ma charakter złożony.

Uzyskane na tym etapie badań wyniki wskazały, że kserotermiczne murawy wapieniolubne złożone są z gatunków o zróżnicowanych wymaganiach zarówno względem odczynu gleby, jak i dostępności żelaza, a zróżnicowanie to można interpretować zarówno w kontekście taksonomicznym, jak i ekologicznym. Funkcjonowanie badanych gatunków roślin preferujących gleby bogate w wapń nie jest uzależnione wyłącznie od dostępności żelaza i odczynu gleby (wpływającego na dostępność żelaza), ale od ilościowych stosunków w dostępności żelaza i manganu modyfikowanych przez wszystkie czynniki edaficzne regulujące dostępność tychże pierwiastków.

W niniejszej części pracy doktorskiej zastosowano jedną metodę doświadczalną (częściowo kontrolowane doświadczenie polowe) oraz pięć technik analitycznych (metody wagowe, laserowy skaning optyczny, spektrofluorymetria, spektrofotometria oraz spektroskopia absorpcji atomowej). W oparciu o uzyskane wyniki opublikowano autorską pracę w czasopiśmie *Geoderma* zatytułowaną „*Differences in iron acquisition strategies of calcicole plant species from xerothermic grasslands*".

P–1

"Differences in iron acquisition strategies of calcicole plant species from xerothermic grasslands"

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Differences in iron acquisition strategies of calcicole plant species from xerothermic grasslands

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ABSTRACT

Little is known about iron (Fe) acquisition of calcicole species from xerothermic grasslands and their resistance to Fe-dependent chlorosis. In this study an experiment was designed to investigate effects of opposing soil types (acidic Podzol vs slightly alkaline Rendzina) and addition of HBED-chelated Fe (five doses of 5 and 25 μmol kg^{-1} soil every two weeks). Selected calcicole plant species occurring on xerothermic grasslands, *Aster amellus* L. (Aa), *Betonica officinalis* L. (Bo), *Prunella grandiflora* (L.) Scholler (Pg), *Salvia verticillata* L. (Sv) and *Veronica teucrium* L. (Vt), were tested in a pot experiment under field conditions. Responses of plants were described using measurements of chlorosis, growth, chlorophyll content and fluorescence, activity of antioxidant enzymes and content of elements, including Ca, Mg, Fe, Mn, Zn and Cu. Some Podzol-grown plants showed arrested growth (up to 80% significant reduction of root and shoot fresh weight (FW) compared to Rendzina-grown plant, depending on species) but no signs of Fe-dependent chlorosis, whereas the same species grown on Rendzina performed very differentially. Lime chlorosis on Rendzina was induced in Aa, Bo and Pg (c.a. 30–40% significant reduction of chlorophyll content compared to Fe-supplied plants) but not in Sv and Vt. Fe-HBED treatment totally diminished chlorosis in species-dependent dose (5 μmol kg^{-1} soil for Aa and Pg and 25 μmol kg^{-1} soil for Bo) but significantly slowed growth (up to 50% significant reduction of root FW and 65% reduction of shoot FW compared to non-Fe-treated Rendzina-grown plant, depending on species). Chlorosis negatively affected functioning of photosynthetic apparatus alternating quantum yield of primary PSII photochemistry (F_V/F_M), light energy dissipation (DI_0/RC) and performance index (PI_{ABS}) and Fe-HBED significantly alleviated these perturbations. Activity of antioxidant enzymes suggested Fe-HBED-caused alleviation of oxidative stress. Reduction of chlorosis did not rely on improved Fe accumulation, nor on altered partitioning on root-shoot axis, but a relation between chlorosis and manganese (Mn) in roots was observed. The results seem to indicate more complex interaction between Fe and Mn in induction of soil-dependent chlorosis than one might expect. Availability of Fe on the slightly alkaline soil limited functioning of the studied calcicole species in specific manner and probably created micro habitats. Ultimately, it can be clearly seen that xerothermic grasslands are composed of chlorosis-resistant and chlorosis-susceptible species.

1. Introduction

It is well established that the chemico-physical characteristics of the soil are important factors that strongly control plant distribution and growth. It is believed that, whilst calcicole plants are able to take up nutrients when grown in calcareous soils (Zohlen, 2002; Bothe, 2015), growth of calcifuge species under high pH (alkaline soils) is limited by low nutrient availability, including phosphorus (P; Tyler, 1992; Zohlen and Tyler, 1997), iron (Fe; Zohlen and Tyler, 1997; Zohlen, 2002), and manganese (Mn; Messenger, 1986; Kuster et al., 2013). This means that under these conditions, relatively low amount of soluble nutrients might be available for some species to uptake and therefore for use in various physiological and metabolic processes, growth, competition and survival of plants.

Xerothermic grasslands, composed of calcicole species, are associated with calcareous soils developing on bedrock rich in calcium (Ca)

Abbreviations: H^+, proton; LHC, light-harvesting complex; PSII, photosystem II; RC, photosynthetic reaction center; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; IDC score, iron deficiency chlorosis score

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(Shimwell, 1971; Poschlod et al., 1998). According to the FAO soil taxonomy (IUSS Working Group WRB, 2015), this kind of soil is classified as Rendzic Leptosol, a soil with low thickness and extremely rich in coarse fragments. Depending on the composition of bedrock, previous and local classification systems (e.g. Polish Soil Classification; Marcinek and Komisarek, 2011) distinguished two general types of Leptosols: Rendzinas (based on calcareous rocks; herein after Rendzic Leptosol are referred to as Rendzina) and Rankers (based on non-calcareous rocks) (IUSS Working Group WRB, 2015). Therefore, Rendzina originating from limestone contains calcaric material rich in calcium carbonate which implies high content of available Ca and neutral or alkaline pH (in contrast to Rendzinas originating from gypsum which are susceptible to acidification) (Leuchner and Ellenberg, 2017). Depending on weathering processes, humus content, biological activity, local vegetation and soil-forming factors, availability of other elements may vary, but it is concerned that well developed Rendzinas are relatively rich in nutrients, meet requirements of nutrient-demanding species and allow development of species-rich communities (Bothe, 2015; Leuchner and Ellenberg, 2017; Kabała, 2018). On the other hand, it must be highlighted that high pH of Rendzina makes some elements such as Fe unavailable to plants due to re-crystallization of ferrihydrite to more crystalline oxides (Barton and Abadía, 2006).

Many plant species develop physiological responses under Fe deficiency which increase Fe uptake. In general, it is established that plants evolved two major strategies of Fe acquisition: Strategy I (characteristic of dicots and non-graminaceous monocots) based on reduction of Fe^{3+} to Fe^{2+} and intake of the latter, and Strategy II (characteristic of monocots) in which Fe^{3+} is chelated by strong ligands – phytosiderophores (reviewed by Colombo et al., 2014). Ability to decrease the rhizospheric pH (acidification) through the activation of plasma membrane-localized P-type H^+-ATPases (Kobayashi and Nishizawa, 2012), which leads to solubilization of Fe-containing compounds, is crucial for Fe acquisition in Strategy I species. Plants utilizing Strategy I support their Fe scavenging also by production of ligands binding Fe^{3+}, namely organic (carboxylic) acids and phenolic compounds (Venturas et al., 2014; Sisó-Terraza et al., 2016). However, the plants utilizing Strategy I (in contrast to Strategy II plants) acquire Fe as soluble Fe^{2+} which means that the pool of Fe-binding ligands remains in soil solution and can further accelerate Fe mobilization (Colombo et al., 2014). Due to the diversity of Fe acquisition strategies, it may be expected that even plant species coexisting in one community differ in individual Fe requirements, efficiency of Fe prospecting/scavenging and ability to survive in communities. It was also showed that diversified requirements of crucial nutrients (probably including Fe) of single species are factors maintaining species richness in calciferous dry grasslands (reviewed by Leuchner and Ellenberg, 2017).

Fe-dependent chlorosis in plants is an old worldwide problem especially occurring in areas of calcareous and/or alkaline soils (Mengel, 1994). Classic studies revealed that species of xerothermic grasslands showed chlorotic phenotype in natural conditions (calcareous soils), namely lime chlorosis syndrome (Grime and Hutchinson, 1967; Hutchinson, 1970). Interestingly, not all individuals from a given species develop chlorosis symptoms in comparable types of environment (Grime and Hutchinson, 1967). It can be explained by mosaic structure of environment composed of microhabitats with different water and heat storage capacity, pH and/or abundance of nutrients and other interacting compounds (Hansen et al., 2006; Leuchner and Ellenberg, 2017). Thus, total amount (depending on site location, sampling depth and soil fraction – Singer et al., 1998) and availability of Fe in Rendzina type soils also vary considerably. Furthermore, it is believed that acidophilic (calcifuge) species are less chlorosis-resistant than plants with strong requirement for alkaline pH of substratum (Grime and Hutchinson, 1967; Hutchinson, 1970; Bothe, 2015).

Lime chlorosis is in fact Fe-deficiency-dependent chlorosis caused by several factors acting simultaneously and it is a major nutritional failure on calcareous soils (Abadía et al., 2011). Although Fe is the fourth most abundant element in Earth's crust (6.3%; Frey and Reed, 2012), its amount in soil solution is extremely low, especially in calcareous soils (the concentration range of Fe^{3+} c.a. 10^{-10} M in soil solution; Bothe, 2015). As Fe predominantly exists in soils in its unavailable forms (hydroxides, oxyhydroxides and oxides), plants cannot absorb it under various environmental conditions such as high soil pH. Thus, it may be expected that at least some plant species associated with calcareous soils are not very efficient in supporting many essential metabolic and physiological processes. Deterioration of Fe acquisition and thus worsening of physiological processes in which Fe participates as a vital element (including synthesizing and maintaining chlorophyll) reduces survival capacity and reproduction ability of a plant organism (Venturas et al., 2014). Such Fe deficiency often has an impact on plant phenotype – tissues located near veins remain green, while the remaining leaf lamina becames more or less chlorotic. Interestingly, total Fe content in a chlorotic leaf may be higher than in a non-chlorotic leaf, but in the former, allocation of Fe is not homogenic – Fe is deposited mainly closely to vascular bundles, while interveinal areas of a leaf lamina has an insufficient content of this element (Abadía et al., 2011; Kumar et al., 2017). It is also well established that Fe is an element with very low or no mobility in plant tissues, because Fe ions are rapidly incorporated into cellular structures (Hansen et al., 2006). Thus, lime chlorosis can be observed mostly on young leaves after Fe depletion or destabilization of Fe supply.

Fe fertilization industry is strongly advanced mostly due to agronomic demands, especially for horticulture (Venturas et al., 2014). It resulted in development of many synthetic Fe chelators such as ethylenediamine-*N,N′*-bis(2–hydroxyphenylacetic) acid (*o,o*-EDDHA), *N,N′*-bis(2-hydroxy-5-methylphenyl)ethylendiamine-*N,N′*-diacetic acid (HJB) and *N,N′*-bis(2-hydroxyphenyl)ethylendiamine-*N,N′*-diacetic acid (HBED) (López-Rayo et al., 2009). Among them, HBED shows the highest capacity to chelate Fe selectively in high pH, both in *in vitro* biochemical tests and in soil (López-Rayo et al., 2009). This property makes HBED a chelator with wide agronomical use, but also allows to study biochemical and physiological responses of calcicole plant species, where HBED can be used as a tool.

Although calcicole species (mostly from the xerothermic grasslands of *Festuco-Brometea* Br.-Bl. et Tüxen ex Soó 1947 class) were studied at the individual as well as at the community level (Mucina and Kolbek, 1993), not much attention was paid to their Fe nutrition (as indicated by Bothe, 2015). For example, it is not known if dicotyledonous plants associated with calciferous soils have low Fe requirements or are able to uptake this element efficiently. It also remains unknown what nutritional, biochemical and physiological effects are associated with lime chlorosis. Thus, the aim of this study was to evaluate responses of five calcicole species form xerothermic grasslands (Table 1) to soil type and Fe-HBED treatment using a comparative setup. The objectives of this study were to test the following hypotheses: 1) calcicole plants have similar requirements for Fe and react similarly when grown on slightly alkaline soil; 2) Fe-dependent lime chlorosis can be alleviated by treatment with HBED-chelated Fe; 3) calcicole plants, when grown on calcareous soil, absorb less Fe than the same plants forced to develop on acid non-calcareous soil with naturally higher contents of soluble Fe; Therefore, the following questions were addressed: 1) is lime chlorosis intensity associated with soil pH requirements of individual species?; 2) can chelated Fe (given as Fe-HBED) reduce or eliminate adverse effects of lime chlorosis?; 3) how do plants respond to soils opposing in their characteristics (acidic Entic Podzol vs slightly alkaline Rendzic Leptosol)?

2. Material and methods

2.1. Soil collection and analysis

The soil samples used in this experiment originate from the natural locations in central Poland. The collected Rendzina represents

Table 1
List of the species used in this study, their seed size, seed mass and centre of abundance. Nomenclature of studied taxa follows The Plant List (2013); [a]seed size follows Bojňanský and Fargašová (2007); [b]seed mass measured in this study (determined by weighing 100 air-dried seeds, expressed as mean ± SD; n = 4).

Species	Abbreviation	Family	Growth form	Seed size [mm][a]	Seed mass [mg][b]	Centre of abundance of adult individuals
Aster amellus L.	Aa	Asteraceae	perennial herb	3.4–3.8 × 1.5–1.8	0.955 ± 0.026	xerothermic grasslands, edges of bushes and copses
Betonica officinalis L.	Bo	Lamiaceae	perennial herb	2.7–3.1 × 1.3–1.5	1.010 ± 0.058	xerothermic grasslands, xerothermic mixed oak woods, thermophilic scrub, meadows, balks
Prunella grandiflora (L.) Scholler	Pg	Lamiaceae	perennial herb	1.7–1.9 × 1.5–1.7	0.813 ± 0.091	xerothermic grasslands
Salvia verticillata L.	Sv	Lamiaceae	perennial herb	1.6–2.0 × 1.1–1.3	0.430 ± 0.044	xerothermic grasslands, thermophilic shrubs in forest edge, brushwood
Veronica teucrium L.	Vt	Plantaginaceae	perennial herb	1.3–1.6 × 1.1–1.2	0.340 ± 0.008	xerothermic grasslands and scrub

substratum required for development of species from the studied plant community. Rendzina was collected near to Winnica reserve in the vicinity of Widawa (51° 26′ 11″ N, 18° 50′ 01″ E) in where persistence of xerothermic grassland (*Festuco-Brometea* class) can be observed. Podzol was collected from the vicinity of Zamość (51° 27′ 32″ N, 18° 59′ 24″ E), where suboceanic pine forest (*Leucobryo-Pinetum* W. Mat. (1962) 1973 association, *Vaccinio-Piceetea* Br.-Bl. 1939 class) grows. This kind of soil is also suitable for development of psammophilous grasslands (*Spergulo morisonii-Corynephoretum canescentis* (Tx. 1928) Libb. 1933 association), a community opposing in its requirements for soil pH to grasslands of *Festuco-Brometea* class. The soil samples were collected from randomly selected sites (c.a. 25 m^2) after removing a litter layer. Each collected soil sample represented 0–20 cm soil layer which corresponds to natural soil microhabitat of the studied species. The soils used for plant cultivation were passed through 5 mm soil sifter. The soil samples to be analyzed were air dried and sieved using 2 mm sieves made of stainless steel. All soil analyses were conducted by Regional Chemical and Agricultural Station in Łódź according to certified norms and procedures used therein (Table 2). The samples were analysed for soil texture, acidity (pH), organic carbon (C), total nitrogen (N) and content of available nutrients, including phosphorus (P), potassium (K), magnesium (Mg), manganese (Mn), copper (Cu), zinc (Zn), iron (Fe) and calcium (Ca). Soil texture class was estimated using textural triangle recommended by United States Department of Agriculture (Soil Science Division Staff, 2017) and Soil Science Society of Poland (Marcinek and Komisarek, 2011). Soil nomenclature follows FAO soil taxonomy classification system (IUSS Working Group WRB, 2015).

2.2. Species selection, seed material, seedling establishment and growth conditions

The selected species belong to different plant families and are important components of the plant communities associated with thermophilous dry grassland establishing on calcareous soils (mostly from *Festuco-Brometea*; Mucina and Kolbek, 1993). According to Ellenberg et al. (1991), the studied species are tolerant of calcareous soil conditions (herein referred to as calcicole) and do not appear on acid soils (Supplemental Table 1). Mature seeds of the studied species (Table 1) were collected during summer 2017 (the exact time was chosen individually for each species), from plants growing in optimal conditions in the Didactic-Experimental Garden of the Faculty of Biology and Environmental Protection (University of Lodz), central Poland (51° 78′ N; 19° 48′ E). The seeds were manually removed and all damaged, malformed or wrinkled ones were not used for experiments. Then, the seeds were stored for 10 days in paper bags in darkness at room temperature (21 °C), avoiding high humidity. The seeds were dry-stratified at 4 °C without any source of light for 10 weeks. The stratified seeds were weighed (four replicates of 100 seeds) and sown in standard 103-well trays (well capacity of 20 cm^3) in Podzol (resulting seedlings were used for planting on Podzol) or in Rendzina (resulting seedlings were used for planting on Rendzina). Germination procedure was conducted under light, at stable temperature of 21 °C and with optimal water supply. The seeds of all used species were of high viability and final germination rate of each species was > 75%. The seedlings (all with similar and representative size within a group) were planted in 1.5 dm^3 pots filled with Podzol or Rendzina. Then, the plants were cultivated in the Didactic-Experimental Garden of the Faculty of Biology and Environmental Protection (University of Lodz) under field conditions with full sun exposure to simulate natural habitat. The local climate is temperate, and the seasons are clearly differentiated. Climatic information pertaining to the period of experiment (1st April to 1st October 2018) is based on the data of the Institute of Meteorology and Water Management – National Research Institute (Warszawa, Poland) and presented in Supplemental Fig. 1. The average lowest temperature during experiment was 13.0 °C (April), and the average high temperature was 20.0 °C (August). Total precipitation during the

Table 2
Properties of soils used in this study. [a]soil type follows classification system of IUSS Working Group WRB (2015) and names given in parentheses indicates in-text reference; * – present, but below the detection threshold of used method; n.d. – not detected.

Attribute		Soil type[a]		Analytical method	Analytical procedure
		Entic Podzol (Podzol)	Rendzic Leptosol (Rendzina)		
soil texture [% of particles with indicatedsize expressed in mm]	2.0–1.0	n.d.	0.04 ± 0.01	laser diffraction	PB 34 ed. 1 (3 July 2006)
	1.0–0.10	97.35 ± 9.74	60.02 ± 6.00		
	0.10–0.05	1.10 ± 0.11	7.70 ± 0.77		
	0.05–0.02	0.92 ± 0.20	9.15 ± 2.01		
	0.02–0.002	0.63 ± 0.14	18.15 ± 4.00		
	< 0.002	n.d.	4.95 ± 1.68		
acidity in KCl (pH)		4.3 ± 0.2	7.3 ± 0.3	potentiometric	PN-ISO 10390:1997
Organic C [%]		0.23 ± 0.05	2.66 ± 0.48	titrimetric	PB 29 ed. 1 (7 September 2004)
Total N [%]		0.021 ± 0.003	0.220 ± 0.031	titrimetric (Kjeldahl method)	PB 49 ed. 2 (1 February 2007)
Mean C:N ratio		10.95	12.01		
Mean N:P ratio		0.38	4.78		
Concentration of available nutrients [mg kg^{-1} soil]	P (P_2O_5)	56.0 ± 8.0	46.0 ± 7.0	spectrophotometric	PN-R-04023:1996
	K (K_2O)	< 10.0*	246.0 ± 36.0	atomic emission spectroscopy	PN-R-04022:1996 + Az1:2002
	Mg (Mg^{2+})	37.0 ± 6.0	94.0 ± 15.0	atomic absorption spectroscopy	PN-R-04020:1994 + Az 1:2004 p. 4
	Mn (Mn^{2+})	< 10.0*	88.1 ± 13.2	atomic absorption spectroscopy	PN-R-04019:1993 p. 5
	Cu (Cu^{2+})	n.d.	1.8 ± 0.3	atomic absorption spectroscopy	PN-R-04017:1992 p. 4
	Zn (Zn^{2+})	n.d.	< 2.5*	atomic absorption spectroscopy	PN-R-04016:1992
	Fe (Fe^{3+})	503 ± 86	414 ± 70	atomic absorption spectroscopy	PN-R-04021:1994 p. 4
	Ca (Ca^{2+})	< 40*	3415 ± 649	atomic emission spectroscopy	PB 04 ed. 1 (21 May 2004)

experiment was 324.9 mm with a minimum in June and maximum in July. During cultivation, the plants were optimally watered and eventually hand-weeded depending on needs to maintain optimal growth conditions.

2.3. Experimental design and Fe-HBED chelate treatment

After one week of acclimatization, the seedlings from Rendzina were subjected to treatment with Fe chelated by N,N′-bis(2-hydroxyphenyl)ethylendiamine-N,N′-diacetic acid (with Fe:chelator ratio of 1:1, Bergeron et al., 1998; López-Rayo et al., 2009) (Fe-HBED, ADOB®, Poland). The plants were treated with 0, 5 or 25 μmol Fe-HBED kg^{-1} soil (50 cm^3 containing appropriate amount of Fe-HBED per pot, prepared with tap water used for routine watering in order to avoid osmotic stress). The seedling from Podzol were supplied with 50 cm^3 of tap water just as those subjected to 0 μmol Fe-HBED kg^{-1} soil treatment (Rendzina alone). The plants were treated every two weeks with appropriate solution (5 doses during the experiment). The experiment were started at 1st April (date of seed sowing) and terminated at 1st October (day of data/plant collection).

2.4. Determination of leaf morphometrics and plant growth parameters

For leaf morphometric measurements, a CI-202 portable laser area meter (CID Bio-Science, USA) was mounted. The petioles of fresh leaves were carefully cut off and each leaf blade was scanned individually. Width, lenght, length/width ratio, area and perimeter were measured using four fully developed leaves per plant and four plant per treatment. To determine the growth, both fresh (FW) and dry weight (DW) of roots and shoots were determined by weighing and drying the plant material in oven in 60 °C for c.a. 48 h until a constant mass was obtained. Allocation of FW and DW was calculated as shoot:root ratio. Fresh and dry weight as well as weight allocation were determined using four plants per treatment.

2.5. Determination of chlorosis using chlorophyll meter and visual scoring

Chlorosis scoring was conducted using non-destructive measurements of chlorophyll content and iron deficiency chlorosis (IDC) scoring. The portable chlorophyll content meter CCM-300 (Opti-Sciences Inc., USA) was used for chlorophyll content determination according to the instruction of the manufacturer. The chlorophyll fluorescence ratio (F_{735}/F_{700}) was measured according to the method of Gitelson et al. (1999) and then deciphered as chlorophyll content in mg m^{-2} using standard equations of the device. Chlorophyll content was measured using four fully developed leaves per plant and four plants per treatment.

The visual scoring of chlorosis (IDC score) was used according to Wang et al. (2008), with slight modifications. The following 1–5 point scale was applied, where: 1 – no chlorosis (green leaf blades), 2 – slight yellowing of leaves, 3 – interveinal chlorosis (green veins and yellow interveinal areas), 4 – interveinal chlorosis and developing necrosis, 5 – severe chlorosis (yellow veins and interveinal areas) and necrosis. For each group, four plants were used for chlorosis scoring.

2.6. Determination of chlorophyll a fluorescence

The changes in polyphasic rise in chlorophyll *a* fluorescence (OJIP) transient were measured using a pulse amplitude modulation fluorometer, FluorPen FP100 (Photon System Instruments, Czech Republic). Measurements were conducted at optimal weather conditions (temperature > 18 °C, cloud cover below 25%, no rainfall). In order to initiate opening of all reaction centers and to minimize fluorescence resulting from energization of the thylakoid membrane, the leaves were dark-adapted for 20 min prior to analysis using standard detachable clips. The distance between the detector and the surface of

leaves was constant for all evaluations. The data were captured using the pre-programmed protocol of the device and the OJIP parameters were calculated according to Strasser et al. (2004) using equations presented therein (Supplemental Table 2). The OJIP parameters were measured using two fully developed leaves per plant and four plants per treatment. Fluorescence fingerprint of the measured parameters (Supplemental Table 2) were presented as means of values normalized to the values measured on the plants grown on Rendzina using radar plot (Berger et al., 2007; Banks, 2017).

2.7. Determination of enzymatic activities

Enzymatic activity was measured in total enzymatic extracts (thus containing cytosolic, apoplastic and ionically-bound proteins; Li et al., 1989; Verma et al., 2008). Leaf and root samples (0.2 g each) were homogenized in chilled mortars with 1 M NaCl in 0.066 M phosphate buffer (pH 7.0) containing 1 mmol EDTA, 1 mmol sodium ascorbate and 1% (w/v) PVP (polyvinylpyrrolidone) (tissue/fluid ratio of 1:10, w/v). The homogenates were centrifuged for 10 min at 7000 rpm (4500 × *g*) at 4 °C. The pellet was discarded and the supernatants were used for further analysis.

Activity of superoxide dismutase (SOD, EC 1.15.1.1) was measured spectrophotometrically using the method of Beauchamp and Fridovich (1971). The reaction mixture contained 0.066 M potassium phosphate buffer (pH 7.8), 13 mmol methionine, 75 μmol NBT, 2 μmol riboflavin, 0.1 mmol EDTA and the enzyme extract. The reaction was started by turning on an UV lamp and increase in absorbance was measured 10 min after start (λ = 560 nm). The activity of SOD was expressed in units, each representing an amount of enzyme required to inhibit 50% of the photochemical reduction of NBT, per minute per mg protein. Activity of catalase (CAT, EC 1.11.1.6) was measured spectrophotometrically by the method of Dhindsa et al. (1981). The reaction mixture contained 0.066 M potassium phosphate buffer (pH 7.0), 15 mmol H_2O_2 and the enzyme extract. The reaction was started by addition of H_2O_2 and decrease in absorbance was measured for 10 min (λ = 240 nm; ε = 36.1 mM^{-1} cm^{-1}). The activity of CAT was expressed in units, each representing 1 mmol of H_2O_2 decomposed, per minute per mg protein. Activity of peroxidase (POD, EC 1.11.1.7) was measured spectrophotometrically by the method of Maehly and Chance (1954). The reaction mixture contained 0.066 M acetate buffer (pH 5.6), 5 mmol guaiacol, 15 mmol H_2O_2 and the enzyme extract. The reaction was started by addition of H_2O_2 and increase in absorbance was measured after 4 min (λ = 470 nm; ε = 26.6 mM^{-1} cm^{-1}). The activity of POD was expressed in units, each representing 1 μmol of tetraguaiacol formed, per minute per mg protein. Protein amount was measured in enzymatic extracts according to the method of Bradford (1976) using bovine albumin as a standard. Enzymatic activity and protein content were measured using four plants per treatment.

2.8. Determination of mineral composition of plant tissues and partitioning of elements

The dried plant material (roots and shoots), which were used to determine FW and DW, were wet-digested in 140 °C in a mixture of HNO_3 and $HClO_4$ (ratio of 4:1, v/v). Concentrations of calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) were determined by atomic absorption spectrometer, SpectrAA 300 (Varian Australia Pty. Ldt., Australia), equipped with an air/acetylene flame and deuterium lamp for background correction. Concentrations of Ca (422.7 nm), Mg (285.2 nm), Fe (248.3 nm), Mn (279.5 nm), Zn (213.9 nm) and Cu (324.8 nm) were measured in whole samples. The atomic absorption standards of elements (BAKER ANALYZED™, J.T. Baker) were used for device calibration. The content of each element was expressed in mg g^{-1} DW or μg g^{-1} DW, depending on results. Contents of all elements were measured in roots and shoots of four plants per treatment.

To determine transportation of nutrients on root-shoot axis, element partitioning was calculated as shoot allocation percentage (SAP) using the following formula (based on the method of Sankaran and Grusak, 2014):

$$SAP = \frac{total\ shoot\ element\ content}{total\ shoot\ element\ content + total\ root\ element\ content} \times 100\%$$

The SAP was expressed in percents (%) and presented as a heatmap of mean values from four plants per treatment.

The contents of Fe and Mn determined in the roots and shoots were used to calculate Fe:Mn ratio.

2.9. Statistical analysis

The experiment was set up in a completely randomized block design. Normality of the data was checked using Kolmogorov-Smirnov's test and homogeneity of variances was checked using the Brown–Forsythe's test (all data met requirements for ANOVA analysis). The effect of treatment (p, r, r5 or r25) on each parameter was analyzed using one-way ANOVA followed by Tukey's HSD post-hoc test. Differences were concerned as significant at value of $p < 0.05$. To test the influence of species (Aa, Bo, Pg, Sv or Vt), experimental treatment (p, r, r5 or r25) and their interaction on the measured parameters, two-way ANOVA was used (differences were accepted as significant at $p < 0.05$). All statistical evaluations were conducted using Statistica 12.0 software (StatSoft Inc., USA).

3. Results

3.1. Soil properties

Podzol was mostly composed of sand particles; silt particles constituted a small percentage and no clay fraction was detected (Table 2). Rendzina contained definitely higher quantity of silt particles at the cost of sand fraction. Furthermore, c.a. 5% of clay was detected in this soil (Table 2). According to these results Rendzina was sandy loam, whilst Podzol was sand.

Soil texture was reflected in mineral composition of the studied soils. Content of P did not differ greatly between the tested soils, however Rendzina contained greater quantity of almost all the other assayed elements (Table 2). Surprisingly, Fe content in Podzol was just slightly greater than in Rendzina. Rendzina was definitely more N- and C-rich than Podzol. According to the calculated C:N ratio, both soils showed similar degree of mineralization. Calculated N:P ratio suggested N-dependent limitations for plants in Podzol soil. As expected, acidity of Podzol (pH = 4.3 ± 0.3) was definitely greater than that of Rendzina (pH = 7.3 ± 0.3).

3.2. Plant performance – development of chlorosis, growth and leaf morphometrics

The experiment showed chlorosis development (both measured by chlorophyll meter and by scoring) on three studied species (Aa, Bo and Pg) when the plants were cultivated on Rendzina (Fig. 1). In these species, chlorosis was not observed when the plants grew on Podzol and decreasing chlorosis intensity was observed when they were subjected to increasing treatment of Fe-HBED (Fig. 1). Two of the studied species (Sv and Vt) performed well in all tested variants which was manifested by no chlorosis symptoms (Fig. 1B). Chlorophyll content and qualitative score of chlorosis were significantly affected by species and the experimental treatment (Supplemental Table 3).

In general, most studied species reacted very similarly to the experimental growth conditions. Some plants cultivated on Podzol (Aa, Sv, Pg) were very small and rachitic which was reflected in low shoot and root FW (Fig. 2). Similar tendency was observed in the shoot and root DW of Vt although these changes were much less pronounced

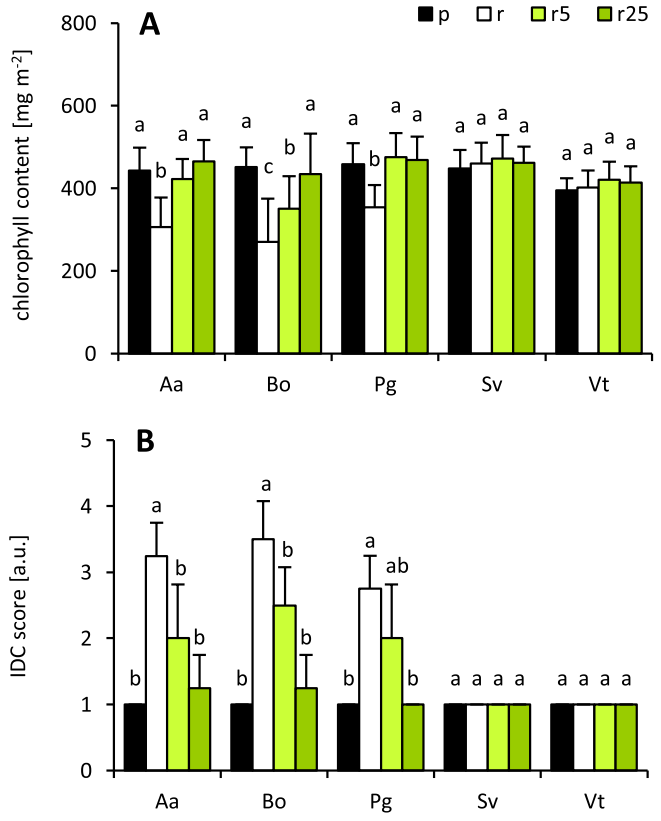

Fig. 1. Chlorosis measured as total chlorophyll content using chlorophyll meter (A) and determined as IDC scoring (B) in the leaves of the studied species grown in Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 μmol kg^{-1} soil Fe-HBED (r25). Values (mean ± SD) with different letters for each parameter are significantly different between the treatments at $p < 0.05$ (n = 16 for A and n = 4 for B; ANOVA with Tukey's HSD post-hoc test).

(Fig. 2). Interestingly, Bo performed similarly in all studied variants but more intensive development of roots was observed when the plants were grown on Podzol (Fig. 2). Significant decreases in shoot and root FW and DW were observed after Fe-HBED application in one studied species (Sv) and two species (Aa and Pg) showed a similar trend although those changes were very subtle (Fig. 2). Interestingly, Fe-HBED promoted FW and DW accumulation in the shoots of Vt. In the case of Bo, Fe-HBED had no greater influence on plant growth except for the significant reduction of shoot growth caused by 5 μmol kg^{-1} soil treatment (Fig. 2).

Analyzing leaf morphometric parameters it can be seen that some plants grown on Podzol had much smaller leaves than those grown on Rendzina – a significant reduction of leaf area was observed in Aa (by 72%), Pg (by 50%) and Sv (by 78%) and this change resulted from both smaller length and width of the leaf blade (Supplemental Table 4). In the case of two species (Bo and Vt) the leaf area remained unaffected (Supplemental Table 4). However, slightly alkaline environment (Rendzina) promoted elongation of leaf blades in Aa and Pg when the plants were compared to those cultivated on acidic soil (Podzol) which resulted in greater length:width ratio (Supplemental Table 4). Compared to Rendzina-grown plants, Fe-HBED treatment promoted leaf size in Aa, Bo and Vt, while in Pg and in Sv reversible trend was observed (Supplemental Table 4).

Comparing the plants cultivated on Podzol and Rendzina, Bo, Pg and Vt grown on Rendzina allocated more FW in shoots than in roots (measured as shoot:root ratio – Supplemental Fig. 2A) but it was counteracted by Fe-HBED treatment (Bo, Pg). Two studied species (Aa and Sv) allocated FW proportionally in all tested variants (Supplemental Fig. 2A). Although some exceptions, allocation of DW corresponded FW shoot:root ratio (Supplemental Fig. 2B).

All parameters referring to plant growth were significantly affected by the species, experimental treatment and their interaction (Supplemental Table 5).

3.3. Fluorescence of chlorophyll a

In general, species highly susceptible to lime chlorosis (Aa and Bo)

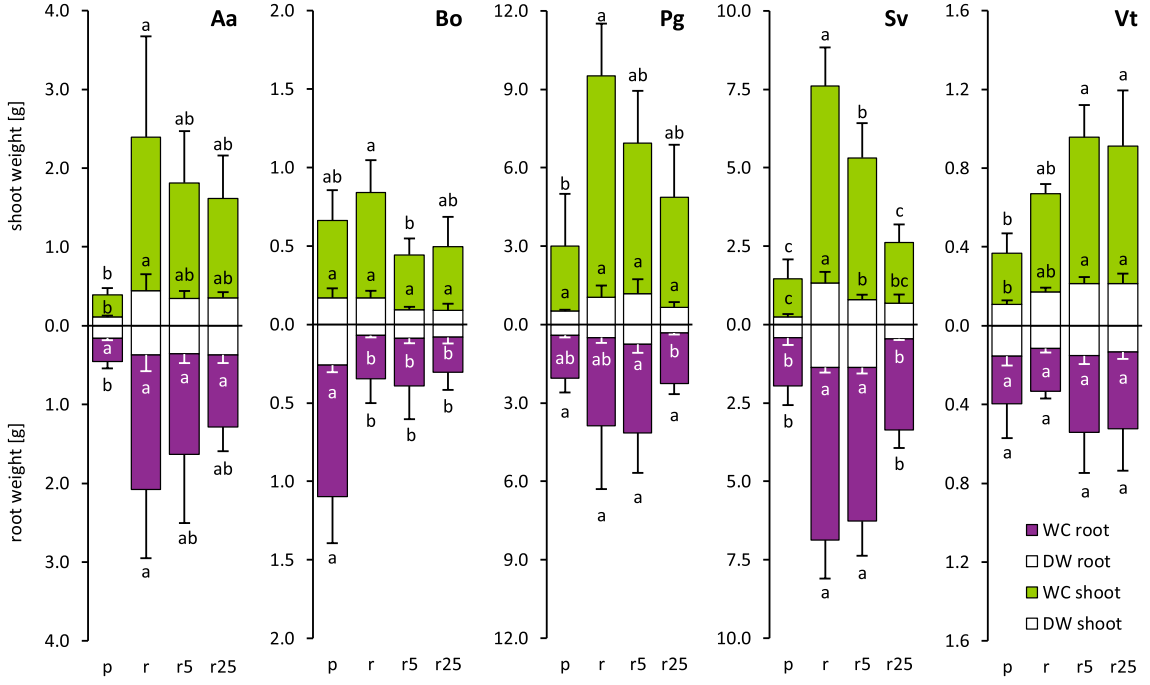

Fig. 2. The effect of Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 μmol kg^{-1} soil Fe-HBED (r25) on fresh weights (FW) and dry weights (DW) of roots and shoots of the studied species. Values (mean ± SD) with different letters for each parameter are significantly different between the treatments at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD post-hoc test). Whole bar – shoot/root FW, transparent bars – shoot/root DW, saturated bars – shoot/root water content (WC).

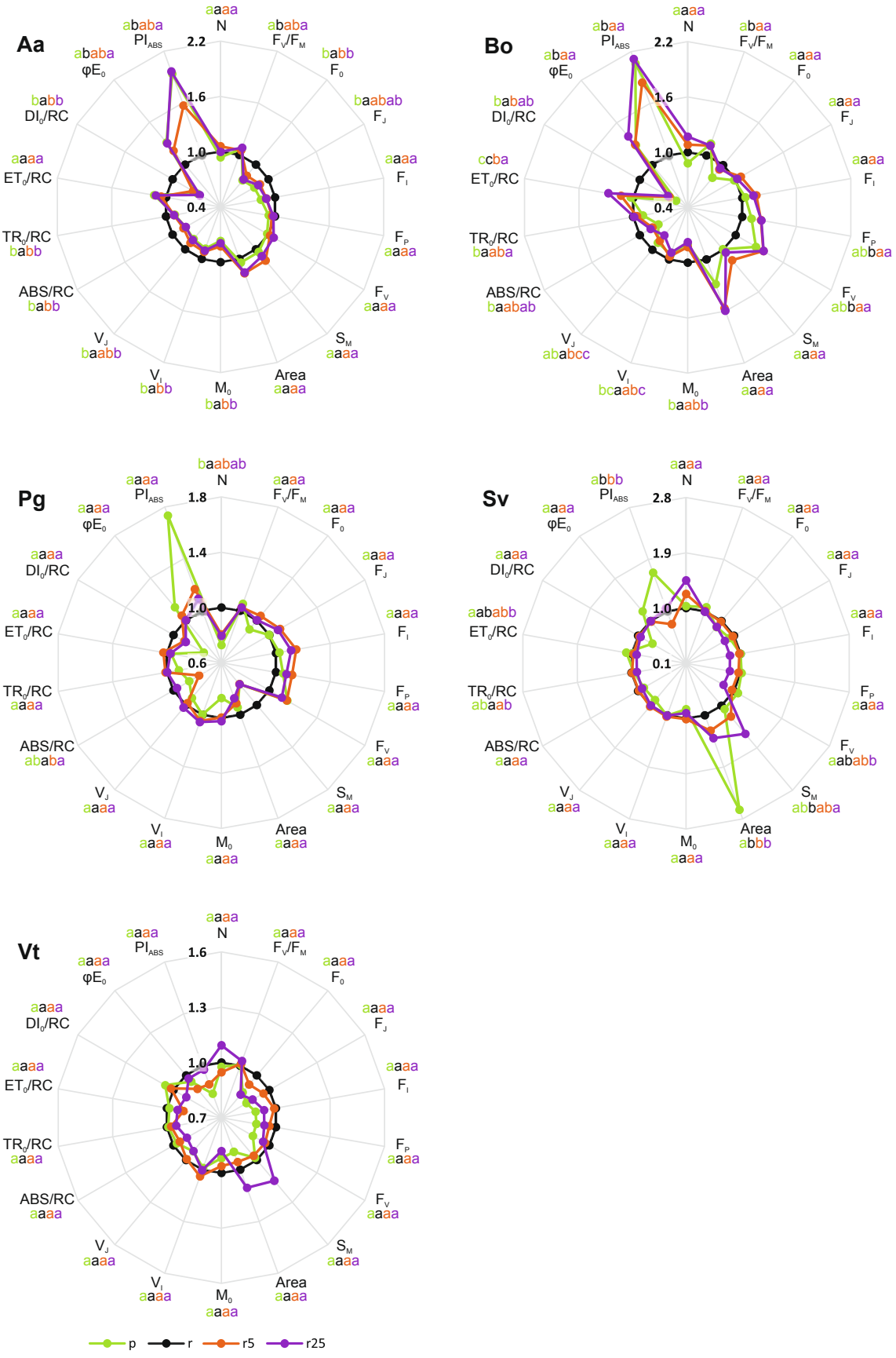

Fig. 3. Relative changes of parameters from chlorophyll *a* fluorescence OJIP transient curves in leaves of the studied species grown on Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 μmol kg^{-1} soil Fe-HBED (r25). Relative values (means) with different letters for each parameter are significantly different between the treatments at $p < 0.05$ (n = 8; ANOVA with Tukey's HSD post-hoc test). The earlier letter indicates higher value of parameter. Color of letter-based statistical indicators refers to each experimental variant as indicated in legend. For interpretation of the references to color in this figure, the reader is referred to the web version of the article.

showed greater alternation of chlorophyll *a* fluorescence-related parameters than the resistant ones (Sv and Vt) (Fig. 3). Interestingly, those parameters in Pg plants (species showing chlorosis) were not as much changed as one might expect (Fig. 3). The Vt plants, which performed well in all experimental variants, did not show any changes regarding the set of OJIP parameters (Fig. 3). Taking into consideration all the studied species and all experimental variants, F_v/F_M, ϕE_0, PI_{ABS}, M_0, ABS/RC, TR_0/RC and DI_0/RC were the most affected OJIP parameters.

When comparing Aa and Bo plants grown on Podzol to respective ones grown on Rendzina, increased values of F_v/F_M, ϕE_0 and PI_{ABS} and decreased values of M_0, V_I, V_I, ABS/RC, TR_0/RC and DI_0/RC (as well as of F_0 and F_J in Aa) were observed in the former ones (Fig. 3). Minor, but significantly lower N values in Pg and higher values of Area and PI_{ABS} (comparing to Rendzina-grown plant) were observed when the Sv plants were grown on Podzol. No changes were detected in Vt plants (Fig. 3).

Comparing the plants grown in Rendzina alone and these grown in Rendzina supplemented with 5 μmol kg^{-1} soil Fe-HBED (Fig. 3), only in Bo treated with chelate increased values of F_P, F_V, F_v/F_M, ϕE_0, PI_{ABS} and ET_0/RC were observed while no increase in any parameters was recorded in the other species. However some decreases were observed in Fe-HBED-treated Aa (F_0, M_0, V_I, ABS/RC, TR_0/RC and DI_0/RC), Bo (V_J) and Pg (ABS/RC) plants while no changes were observed in Vt plants (Fig. 3).

Comparison of Rendzina-grown plants and plants grown on Rendzina with addition of 25 μmol kg^{-1} soil Fe-HBED (Fig. 3) showed that the treatment caused increase in F_v/F_M, ϕE_0, PI_{ABS} in Aa, F_v/F_M, ϕE_0, PI_{ABS}, F_P, F_V and ET_0/RC in Bo and S_M in Sv as well as decrease in F_0, M_0, V_J, V_I, ABS/RC, TR_0/RC and DI_0/RC in Aa, M_0, V_J, V_I in Bo and TR_0/RC in Sv. Two studied species (Pg and Vt) remained unaffected by Fe-HBED treatment of 25 μmol kg^{-1} soil (Fig. 3).

Two-way ANOVA indicated that OJIP parameters were significantly affected by the species (16 of 18), experimental treatment (11 of 18) and their interaction (11 of 18) (Supplemental Table 6).

3.4. Activity of antioxidant enzymes

In many cases, application of Fe-HBED caused alternation of the activity of antioxidant enzymes. Comparing the plants subjected to Podzol and Rendzina, no influence of the soil type on SOD activity in roots were recorded (Fig. 4). Fe-HBED treatment caused different effects depending on the plant species. Activity of SOD in the roots of Bo, Pg, Sv and Vt was not significantly affected (although Bo and Vt showed slight decreasing tendency), while in Aa it was significantly lesser in the plants subjected to 5 μmol kg^{-1} soil Fe-HBED when compared to Rendzina-grown plants (Fig. 4). Lesser activity of CAT in the roots was observed in Aa and Pg cultivated on Podzol than in those cultivated on Rendzina (Fig. 4). The reverse trend was observed in Vt, while soil type had no effect on Bo and Sv (Fig. 4). Fe-HBED treatment caused decrease in CAT activity only in the roots of Aa and Pg, whereas other species remained unaffected (Fig. 4). Comparing Podzol- and Rendzina-grown plants, Aa and Pg and Vt showed lesser activity of POD in roots when the plants were grown on the acidic substratum and this effect was not observed in Bo and Sv (Fig. 4). Fe-HBED caused dose-dependent decrease in POD activity in all studied species except Vt, where decrease was observed just after application of 5 μmol kg^{-1} soil chelate (Fig. 4).

The plants from Podzol did not show changes of SOD activity in leaves in comparison with the plants from Rendzina, except Vt in which increased activity was observed (Fig. 4). Fe-HBED-dose-dependent decrease in SOD activity in leaves was observed just in Aa, while this trait was constant in the other species (Fig. 4). Only two species grown on Podzol (Bo and Pg) showed decreased CAT activity in the leaves compared to Rendzina-grown ones (Fig. 4). Fe-HBED-treated Sv plants showed lesser activity of leaf CAT than those from non-treated Rendzina, while no effect of application was observed in Aa, Bo, Pg (Fig. 4). Plants from Podzol and Rendzina had very similar activity of POD in leaves (except Aa) (Fig. 4). Interestingly, 5 μmol kg^{-1} soil Fe-HBED treatment stimulated POD activity in the leaves of all studied species (54–230% increase compared to non-treated Rendzina, depending on species) and 25 μmol kg^{-1} soil Fe-HBED caused decrease in POD activity below the value of non-treated Rendzina grown Aa and Pg (Fig. 4).

Two-way ANOVA indicated that activities of all assayed enzymes were significantly affected by the species, experimental treatment and their interaction (Supplemental Table 7).

3.5. Mineral composition, element partitioning and relation of iron to manganese

Analysis of microelements showed that each species performed in an

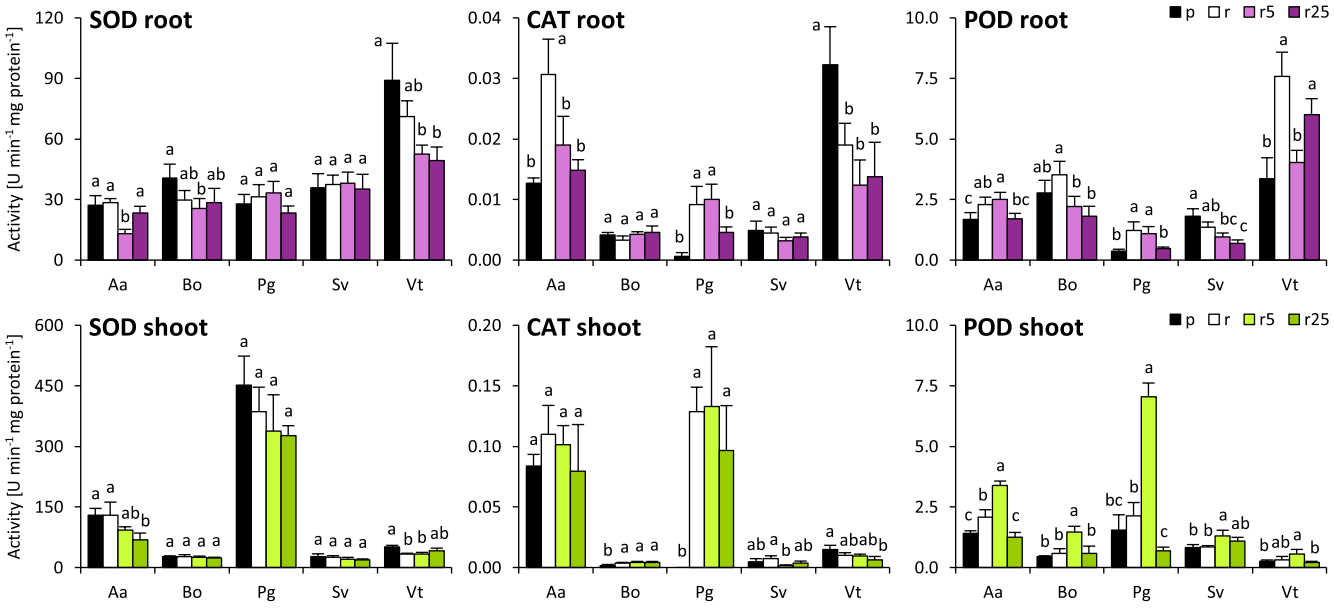

Fig. 4. Activity of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) in roots and shoots of the studied species grown on Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 μmol kg^{-1} soil Fe-HBED (r25). Values (mean ± SD) with different letters for each parameter are significantly different between the treatments at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD post-hoc test).

Table 3
Root and shoot elemental composition of the studied species grown in podzol (p), rendzina (r) or rendzina with addition of 5 μM Fe-HBED (r5) or 25 μM Fe-HBED (r25). Values (mean ± SD) with different letters for each parameter are significantly different between the treatments at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD post-hoc test).

Treatment		Ca [mg g^{-1} DW]	Mg [mg g^{-1} DW]	Fe [mg g^{-1} DW]	Mn [mg g^{-1} DW]	Zn [mg g^{-1} DW]	Cu [μg g^{-1} DW]
		Roots					
Aa	p	4.247 ± 0.786a	3.886 ± 0.678a	0.811 ± 0.158a	0.021 ± 0.003b	0.124 ± 0.027a	7.168 ± 0.971a
	r	6.625 ± 2.051a	2.874 ± 0.207b	0.670 ± 0.091a	0.064 ± 0.016a	0.138 ± 0.142a	3.201 ± 1.485b
	r5	6.456 ± 0.892a	2.427 ± 0.204b	0.696 ± 0.125a	0.070 ± 0.016a	0.077 ± 0.023a	2.209 ± 0.571b
	r25	10.326 ± 7.389a	2.848 ± 0.225b	0.882 ± 0.520a	0.046 ± 0.007ab	0.070 ± 0.026a	2.245 ± 0.568b
Bo	p	15.191 ± 6.766a	2.745 ± 0.240a	2.197 ± 0.346a	0.054 ± 0.007a	0.063 ± 0.005a	4.312 ± 0.639a
	r	6.946 ± 0.972b	2.438 ± 0.386a	0.803 ± 0.127b	0.034 ± 0.005b	0.112 ± 0.032a	2.620 ± 1.252ab
	r5	7.749 ± 3.555ab	2.462 ± 0.291a	0.843 ± 0.386b	0.033 ± 0.011b	0.071 ± 0.013a	1.759 ± 0.653b
	r25	7.576 ± 0.757ab	2.554 ± 0.436a	0.825 ± 0.178b	0.019 ± 0.006b	0.097 ± 0.038a	2.202 ± 0.894b
Pg	p	4.091 ± 0.658b	1.980 ± 0.259a	0.850 ± 0.225a	0.023 ± 0.004b	0.071 ± 0.015b	3.058 ± 1.101a
	r	18.929 ± 8.096a	2.338 ± 0.578a	1.396 ± 0.455a	0.044 ± 0.011a	0.083 ± 0.027ab	2.283 ± 0.655a
	r5	14.286 ± 4.030a	2.423 ± 0.268a	1.222 ± 0.395a	0.036 ± 0.005ab	0.101 ± 0.026ab	2.620 ± 0.966a
	r25	11.504 ± 1.814ab	2.049 ± 0.229a	0.938 ± 0.258a	0.030 ± 0.003b	0.120 ± 0.012a	2.635 ± 0.169a
Sv	p	4.003 ± 0.892b	4.149 ± 0.609a	0.404 ± 0.069ab	0.024 ± 0.004a	0.065 ± 0.014a	3.580 ± 0.821a
	r	17.568 ± 11.691a	5.275 ± 0.890a	0.357 ± 0.075ab	0.013 ± 0.002b	0.072 ± 0.027a	2.222 ± 0.349b
	r5	11.404 ± 5.218ab	5.083 ± 0.322a	0.554 ± 0.254a	0.018 ± 0.006ab	0.067 ± 0.009a	2.411 ± 0.361b
	r25	5.943 ± 0.795ab	5.020 ± 0.693a	0.250 ± 0.034b	0.010 ± 0.001b	0.091 ± 0.049a	2.358 ± 0.108b
Vt	p	9.607 ± 2.741a	2.213 ± 0.101b	1.386 ± 0.289a	0.053 ± 0.010a	0.136 ± 0.007ab	2.927 ± 0.492ab
	r	20.385 ± 8.223a	2.622 ± 0.023a	1.852 ± 0.435a	0.055 ± 0.009a	0.144 ± 0.017a	2.539 ± 0.171b
	r5	19.942 ± 10.540a	2.422 ± 0.310ab	1.621 ± 0.423a	0.047 ± 0.009a	0.132 ± 0.020ab	2.900 ± 0.564ab
	r25	19.558 ± 5.197a	2.583 ± 0.075a	1.797 ± 0.337a	0.047 ± 0.008a	0.104 ± 0.020b	4.229 ± 1.173a
		Shoots					
Aa	p	18.142 ± 1.055a	3.818 ± 0.781a	0.654 ± 0.081a	0.026 ± 0.005b	0.117 ± 0.032a	3.155 ± 1.408a
	r	13.420 ± 2.569a	2.283 ± 0.260b	0.462 ± 0.115b	0.103 ± 0.027a	0.074 ± 0.046a	1.837 ± 0.132ab
	r5	15.373 ± 4.744a	2.493 ± 0.258b	0.467 ± 0.095b	0.111 ± 0.012a	0.119 ± 0.028a	1.454 ± 0.281b
	r25	13.184 ± 2.922a	2.652 ± 0.383b	0.276 ± 0.011c	0.081 ± 0.019a	0.088 ± 0.020a	1.721 ± 0.380ab
Bo	p	24.146 ± 13.788a	2.131 ± 0.244a	1.010 ± 0.883a	0.066 ± 0.012ab	0.041 ± 0.007a	4.375 ± 1.025a
	r	18.348 ± 6.430a	3.051 ± 0.874a	0.502 ± 0.285a	0.052 ± 0.012b	0.062 ± 0.011a	2.019 ± 0.548b
	r5	11.405 ± 1.981a	2.143 ± 0.418a	0.282 ± 0.026a	0.086 ± 0.018a	0.047 ± 0.012a	2.292 ± 0.786b
	r25	15.883 ± 1.121a	2.295 ± 0.199a	0.470 ± 0.156a	0.038 ± 0.012b	0.050 ± 0.013a	1.918 ± 0.734b
Pg	p	12.363 ± 0.700a	5.673 ± 0.245a	0.432 ± 0.083a	0.026 ± 0.004b	0.065 ± 0.011a	3.595 ± 0.478a
	r	21.617 ± 10.742a	4.103 ± 0.290b	1.095 ± 0.748a	0.055 ± 0.008a	0.078 ± 0.041a	2.778 ± 0.755a
	r5	38.688 ± 22.892a	3.873 ± 0.380b	1.212 ± 0.618a	0.038 ± 0.011b	0.091 ± 0.007a	2.491 ± 1.052a
	r25	16.403 ± 8.441a	3.998 ± 0.514b	0.812 ± 0.491a	0.035 ± 0.008b	0.097 ± 0.013a	2.368 ± 0.610a
Sv	p	39.364 ± 13.389a	5.655 ± 1.185a	0.470 ± 0.087a	0.026 ± 0.005a	0.101 ± 0.015a	5.014 ± 1.357a
	r	40.371 ± 10.044a	5.731 ± 1.626a	0.293 ± 0.109b	0.015 ± 0.004b	0.071 ± 0.006b	3.335 ± 0.516b
	r5	25.683 ± 18.275a	8.093 ± 1.523a	0.316 ± 0.061ab	0.015 ± 0.001b	0.075 ± 0.007b	3.348 ± 0.335b
	r25	43.729 ± 5.356a	6.351 ± 1.407a	0.233 ± 0.055b	0.012 ± 0.002b	0.065 ± 0.010b	4.099 ± 0.533ab
Vt	p	17.721 ± 1.947b	2.298 ± 0.192b	0.680 ± 0.115ab	0.026 ± 0.002b	0.098 ± 0.008a	2.256 ± 0.984a
	r	21.311 ± 2.321ab	2.509 ± 0.114b	0.784 ± 0.153ab	0.031 ± 0.002a	0.099 ± 0.014a	1.903 ± 0.274a
	r5	22.002 ± 0.781a	2.952 ± 0.217a	0.588 ± 0.034b	0.025 ± 0.003b	0.097 ± 0.009a	1.765 ± 0.148a
	r25	22.598 ± 1.414a	2.500 ± 0.124b	0.888 ± 0.160a	0.028 ± 0.004ab	0.094 ± 0.008a	2.094 ± 0.447a

individual manner. Regarding the content of elements in roots (Table 3), there was no differences in Ca content between Podzol- and Rendzina-grown Aa and Vt plants, whereas this parameter was significantly greater in Rendzina-grown than in Podzol-grown Pg and Sv plants (Bo showed the opposite trend). Roots of Fe-HBED-treated plants did not contained more Ca than the plants grown on Rendzina (Table 3). There was no significant difference considering Mg content in the roots of all species, except comparison between Podzol- and Rendzina-grown Aa and Vt plants (Table 3). Very surprisingly, the roots of Fe-HBED-treated plants had the same content of Fe as the not-treated ones and only Bo showed enhanced content of this element in roots when forced to grow on Podzol (Table 3). Significant differences between contents of Mn in the roots of plants from Podzol and from Rendzina were observed in almost all species (except Vt) and Pg plants treated with Fe-HBED showed reduced content of this element compared to not-treated ones (Table 3). Content of Zn was very stable among variants within species, except Vt, in which Fe-HBED negatively affected Zn contents in the roots (Table 3). Comparing Podzol- and Rendzina-grown plants, Aa and Sv forced to grow on acidic soil showed higher contents of Cu in the roots than the same species grown on slightly alkaline soil. Fe-HBED affected Cu content in the roots only in Vt (Table 3).

Some changes were also observed in the shoots (Table 3). Soil type and Fe-HBED treatment had no significant influence on Ca content the in the shoots (except Aa and Vt; Table 3). Fe-HBED also did not significantly affected Mg content in shoots, however, Podzol-grown Aa and Pg had higher contents of this element than the plants grown on Rendzina (Table 3). Significantly higher contents of Fe in shoots of Aa and Sv grown on Podzol than in those grown on Rendzina were observed. Very surprisingly, the treatment with Fe-HBED did not enhance significantly Fe content, on the contrary it even caused significant decrease in the content of this element (Aa; Table 3). Fe-HBED treatment caused decrease in Mn content in Pg and Vt (Table 3). Some plants (Aa and Pg) showed lesser content of this element when were grown on acidic soil, compared to slightly alkaline soil. Only Sv showed significant differences in the content of Zn in the shoots (Podzol vs Rendzina; Table 3). Higher contents of Cu in Podzol-grown Bo and Sv plants than in Rendzina-grown ones were observed. Fe-HBED did not

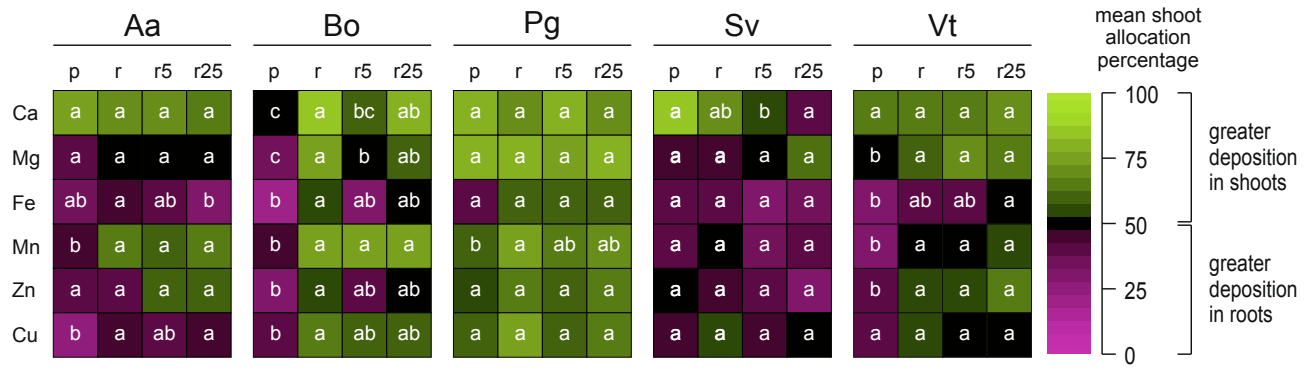

Fig. 5. Heatmap of element partitioning measured as shoot allocation percentage (SAP) in the studied species grown on Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 μmol kg^{-1} soil Fe-HBED (r25). Values (means) with different letters for each parameter are significantly different between the treatments at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD post-hoc test). The earlier letter indicates higher value of parameter. For interpretation of the references to color in this figure, the reader is referred to the web version of the article.

significantly affected Zn and Cu contents in any of the studied species (Table 3).

Two-way ANOVA showed that contents of all elements were significantly dependent on species (Supplemental Table 8). However, the contents of Mg, Fe and Zn in the roots as well as contents of Ca, Mg, Fe and Zn in the shoots did not depend on treatment (Supplemental Table 8). Interactions of the studied factors were significant in almost all cases, except contents of Zn in the roots and Cu in the shoots (Supplemental Table 8).

In general, Aa, Bo and Vt plants forced to grow on Podzol partitioned more microelements such as Mg, Fe, Mn, Zn and Cu into roots (Fig. 5). The Pg plants partitioned all analyzed elements mostly into the shoots in all studied variants, whereas Sv loaded some elements (Fe, Mn, Zn) into the roots (Fig. 5). Fe-HBED affected SAP values only for Ca (Bo, Sv), Mg (Bo) and Fe (Aa) (Fig. 5). Calculated SAP value significantly dependent on treatment, species and interaction of these factors almost for all elements, except influence of species and treatment on Ca and their interaction on Cu (Supplemental Table 8).

Chlorosis-prone species (Aa and Bo) had higher ratios of Fe:Mn in the roots when grown on Podzol than on Rendzina (Fig. 6). Fe-HBED in the dose of 25 μmol kg^{-1} soil significantly increased Fe:Mn ratio in the roots only in Bo, although similar trend was observed in Aa (Fig. 6). No differences in this parameter were showed in Pg. Chlorosis-resistant species (Sv, Vt) grown on Podzol had significantly lower Fe:Mn ratios in comparison to the respective Rendzina-grown plants (Fig. 6). Fe-HBED did not increase Fe:Mn ratio in the plants in which chlorosis was not observed (Fig. 6). This tendency was not observed in shoots with exception to Aa (Fig. 6). Two-way ANOVA showed significant influence of the species, experimental treatment and their interaction on Fe:Mn ratio with exception to influence of experimental treatment on Fe:Mn ratio in shoots (Supplemental Table 9).

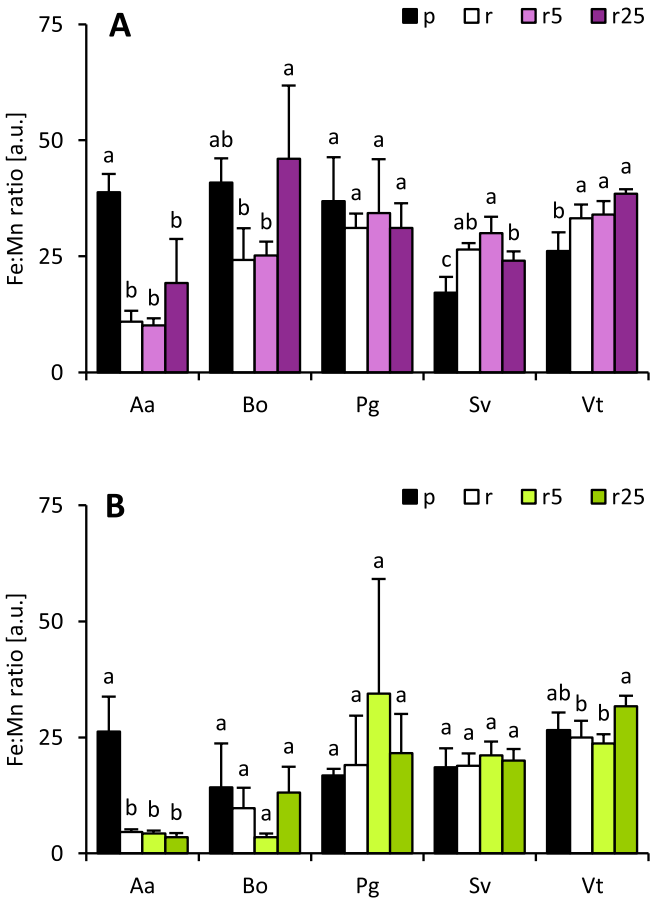

Fig. 6. Iron to manganese (Fe:Mn) ratio in roots (A) and shoots (B) of the studied species grown on Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 μmol kg^{-1} soil Fe-HBED (r25). Values (mean ± SD) with different letters for each parameter are significantly different between the treatments at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD post-hoc test).

4. Discussion

The results showed that Ellenberg indicator value of soil reaction (R) did not correspond with ability of the studied dicotyledonous plant species to avoid lime chlorosis on calcareous soil (question 1), which was in general reported before for some species (reviewed by Bartelheimer and Poschlod, 2016). The presented data denoted also that even plant species originating from the same family (Bo, Pg, Sv) reacted very differentially to Fe status in soil. It is in agreement with conclusion drawn recently for three crop plant species (*Triticum durum* L., *Hordeum vulgare* L. and *Zea mays* L.) from Poaceae family (Celletti et al., 2016a). According to aims of this study, it drives us to put forward a hypothesis (alternative to hypothesis 1) that the studied calcicole plant species have different Fe demands. It is also known that thresholds of Fe content in substratum causing chlorosis are higher than those leading to alternations in Fe nutritional status of plants (Celletti et al., 2016b). Thus, differential response of the plant species recorded in this investigation leads to a conclusion that Fe is an element which strongly shapes habitat of calcareous dry grasslands and creates microhabitats. Similar conclusions were drawn for other environmental conditions, e.g. nitrogen form and its availability as well as water resources, which drived evolution of many physiognomic adaptations that can be observed in calcicole plant species (Leuchner and Ellenberg, 2017).

According to the presented data, addition of chelated Fe diminished or totally relieved chlorosis (question 2) which supports hypothesis 2 and implies that in the studied species the development of chlorosis in calcareous soils is Fe-dependent and may partially results from inability of these species to release Fe from its insoluble form. The studied species have rather low Fe requirements, as five doses of 5 μmol kg^{-1} soil (Aa and Pg) or 25 μmol kg^{-1} soil of HBED-chelated Fe applied every 2 weeks were enough to satisfy Fe demands. On the other hand, it must be mentioned that HBED can remain in soil and continue to solubilize Fe (Kang et al., 2019). It is very probable that at least some calcicole plants are not able to solubilize Fe-containing compounds by local acidification due to high buffering capacity (soil carbonate buffer) of Rendzina type soil (Grillet and Schmidt, 2017). It is also known that species susceptible to lime chlorosis may not always be chlorotic (Grime and Hutchinson, 1967). Combining these two information it can be stated that the final level of chlorosis that can be observed in the field may be due to local differences in Fe availability and complex ecological network that occurs in soil. It was showed in numerous studies that Fe pool was continuously remodeled which resulted from both plant and microbial activity. As an example, available pool of Fe in calcareous soils was showed to be affected by local acidification, root secretion, including production of phytosiderophores, as well as occurrence of siderophores of microbial origin (Colombo et al., 2014; Lewis et al., 2019). All this also implies alternations of other soil nutrients (Ström et al., 1994), which further affect directly and indirectly plant reaction in species-dependent manner. Therefore, performance of plants susceptible to lime chlorosis is at least partially connected with interspecific interactions, which were observed in the field (Tagliavini and Rombolà, 2001) and experimentally evaluated (Cesco et al., 2006; Dai et al., 2019).

Surprisingly, Vt, recognized as a remarkably calcicole species, was able to survive and develop on Podzol soil in a similar rate to the plants grown on Rendzina (which suggests its eurytopic behavior in terms of soil pH requirements), while the other studied calcicoles were not able to maintain normal growth rate when grown on Podzol. It can be hypothesized, that although those species are able to form temporal populations on acidic soils, they are unable to establish permanently and reproduce under such conditions due to very slow growth. Furthermore, it was observed that some plants that grew on Podzol (Bo, Pg and Vt) invested more biomass into roots than shoots while in all studied species the leaves became small and dark green. This mechanism may depend on water holding capacity of soil (Poeplau and Kätterer, 2017) and nutritional limitations, including N and P (Ericsson, 1995; Li et al., 2014) which is very likely since used Podzol had sandy texture, contained about 90% less available N compared to Rendzina and at acidic pH P became less available for root acquisition.

In almost all studied species increasing amount of added Fe-HBED caused growth reduction as it was recorded for plant weight and morphological parameters of leaves. This is in agreement with studies on Fe excess-caused toxicity in sweet potato (*Ipomoea batatas* L.), sisal (*Furcraea hexapetala* (Jacq.) Urb.) and selected dock species (*Rumex crispus* L., *R. maritimus* L. and *R. thyrsiflorus* Fingerh.) (Laan et al., 1991; Adamski et al., 2012; Casierra-Posada et al., 2017). However, the treatment with Fe-HBED was responsible for both stimulation (Aa) or reduction (Pg, Sv) of leaf size and these changes might depend on individual Fe requirements of species. Excess of Fe within plant tissues is believed to be responsible for oxidative stress (due to its pivotal role during Fenton's reaction – Le et al., 2019) which may be responsible for halted growth (at least partially, due to not proportional trade-offs between growth and stress coping mechanisms; Souza and Lüttge, 2015). It is also worth noting, that this effect is not caused by HBED chelator, as similar effects of Fe excess were observed for Fe^{2+}-containing salt ($FeCl_2$ – Laan et al., 1991; $FeSO_4$ – Casierra-Posada et al., 2017) and for Fe^{3+}-EDTA-chelated form (Adamski et al., 2012). From a wider perspective, supraoptimal load of Fe may have ecological impact for persistence of xerothermic grasslands. A hypothesis can be put forward that retarded growth of herbs caused by excess of Fe (e.g. due to root activity of neighboring plants) has detrimental effects on ability to win competition at seedling stage during encroachment of woody species, mostly from xerothermic scrubs and basiphilous thermophilous forests, e.g. *Prunus spinosa* L., *Cornus sanguinea* L., *Ligustrum vulgare* L. and *Ulmus minor* Mill., as their expansion (also on 'vegetation gaps') was listed among natural threat of calcareous xerothermic grasslands in central Europe (Dostálek and Frantík, 2008). On the other hand, leaf enlargement and/or elongation during stress caused by Fe deficiency observed in this study can be paradoxically a beneficial adjustment allowing to win competition for limited resources by altering light conditions, because revegetation of many light-loving species in this environment is connected with efficient scavenging of light (Mortimer, 1992).

The OJIP testing showed that the soil conditions and Fe-HBED treatment induced alternations in the kinetics of the chlorophyll *a* fluorescence. For example, it was observed that some plants (Aa and Bo) grown on Rendzina showed significantly higher value of light energy dissipation (DI_0/RC) and definitely lower performance index (PI_{ABS}) compared to any other variant, which is in total agreement with the study on *Sorghum bicolor* (L.) Moench subjected to Fe starvation (Luna et al., 2018). Similar data were also presented for *I. batatas* subjected to different Fe supply (Adamski et al., 2011). As indicated previously (Luna et al., 2018) changes in PI_{ABS} suggest reduction of energy flux on light-harvesting pigments-RCs axis or disassembly of PSII. This is consistent with the study on *Lactuca sativa* L. subjected to Fe deprivation in which connection of LHCs to inactive PSII was proposed (Msilini et al., 2011). In the same study insufficient Fe supply negatively influenced maximum quantum yield of primary PSII photochemistry (F_v/F_M) which was also observed during presented experiments in the chlorosis-susceptible species (Aa and Bo) and in other plant species, e.g. spinach (*Spinacia oleracea* L.; Timperio et al., 2007). Many similar observations of Fe-dependent inhibition of PSII photochemistry were reported in other higher plants (Larbi et al., 2006 and articles cited therein). Considering molecular basis of this phenomenon, Fe-caused damage of LHCs and RCs may be indirect and direct, either due to disordered chlorophyll biosynthesis (which is supported by our results) or incorrect incorporation of Fe into structures of photosynthetic apparatus (Msilini et al., 2011). On the other hand, such changes in chlorophyll *a* fluorescence were not observed in chlorosis-resistant Sv an Vt and similar mode of action was reported in resistant to Fe-deficiency *Sorghum* × *drummondii* (Nees ex Steud.) Millsp. & Chase (Luna et al., 2018). In summary, alternations in connectivity at PSII caused by Fe deficiency in chlorosis-prone species as well as lack of such changes in resistant ones are very common among many terrestrial vascular plants. Moreover, presented results show that xerothermic grasslands are at least partially a mixture of species able to maintain primary physiological functioning themselves and those that are not able to do it (in terms of Fe nutrition).

Regarding SOD and CAT activity it can be stated that the results depended on species and treatment. However, the results clearly showed connection between Fe-caused alleviation of chlorosis and activity of SOD and CAT – in many cases the greater dose of Fe, the lower activity of SOD and CAT. All Fe perturbations result in photooxidative stress (Le et al., 2019) affecting redox homeostasis by impairments of cellular redox chains (mainly in plastids) (Grillet and Schmidt, 2017) and thus causing constant risk of electron transfer leaks and formation of ROS. Considering chlorophyll *a* fluorescence data and activity of SOD, it seems possible that Fe-HBED treatment relieved oxidative stress in chloroplasts. This is in agreement with studies showing lesser content of H_2O_2 in leaves of sunflower (*Helianthus annuus* L.) plants optimally supplied with Fe than in Fe-starved ones (Ranieri et al., 2001). Furthermore, high availability of H_2O_2 (mM range) is required to trigger activity of CAT (Adamski et al., 2012; Anjum et al., 2016), thus presented results pertaining to activity of CAT (in agreement with SOD activity) suggest limitation of H_2O_2 generation (leading to alleviation of

oxidative stress).

The data pertaining to POD activity are very hard for interpretation regarding multifunctional nature of this enzyme (Passardi et al., 2005) and direct multifactorial regulation of its activity, e.g. by pH and presence of metals (Elsayed et al., 2018). Honestly it can be stated that our results as well as physiological, biochemical (Ranieri et al., 2001) and –omic (Zamboni et al., 2012; Khandakar et al. 2013, López-Millán et al., 2013; Zamboni et al., 2017) studies indicated complex relation between POD activity and Fe, as abundance and/or activity of its isoforms were showed to change dramatically depending on Fe status. Activity of POD in the roots decreased with increasing addition of Fe which denoted that the plants subjected to Fe treatment were less stressed than those that were not supplied with this element. Regarding presented data, marked increase of POD activity in the leaves of plants subjected to 5 μmol kg^{-1} soil HBED treatment may be physiologically attributed to greater availability of phenolic reductants needed for peroxidation. Such a mode of action was presented for potato (*Solanum tubersosum* L.) calli cultures supplied with 5 μM EDTA-chelated Fe (Boamponsem et al., 2017). Furthermore, presented results (r vs r5 comparison) are in agreement with previous reports showing higher activity of POD in leaves of plant with sufficient Fe nutrition compared to those subjected to Fe starvation (Tewari et al., 2005). On the contrary, no linear trend of POD activity was observed in any of the studied species (especially considering the plants treated with 25 μmol kg^{-1} soil Fe-HBED), thus we believe that simplification of proportional changes in Fe content and POD activity should be avoided.

Previous studies strongly suggested non-enzymatic chemical Fe reduction as a major mechanism satisfying Fe demands in plants (especially at alkaline pH), which is connected with root exudation of low-molecular organic acids (LOAs) (Grillet and Schmidt, 2017). For example, root exudates of some calcicole species contain several times more LOAs than exudates of calcifuge species (Tyler and Ström, 1995). However, even among species associated with limestone soils, considerable diversity of exudation pattern can be observed which supports the conception of Fe micro-habitats and explains differences between reactions of studied species. Furthermore, *Veronica spicata* L., a close relative of studied Vt, exudates high quantity of citrate which was showed to solubilize Fe from lime soil 8–50 times more efficiently that other studied LOAs (Tyler and Ström, 1995). This partially explains why symptoms of lime chlorosis were not observed in this species and why content of Fe in Vt roots was 25–80% greater than in any other studied species. Regarding Fe acquisition, the differences between all tested variants among a given species were definitely lesser than one might expect. As significant differences in contents of this element between Podzol- and Rendzina-grown plants were observed just in very few cases (Bo, Sv and Vt), hypothesis 3 is probable only for selected species tested in this study. According to this, it can be stated that the studied species react very differentially to soils opposing in their characteristics (question 3).

Presented elemental partitioning data support the common hypothesis that lime chlorosis is caused by inadequate Fe allocation within leaf blade due to its unsettled Fe import from apoplast to symplast, but not by impaired transport on root–shoot axis. It is in agreement with a recent study showing that chlorotic leaves of Fe-starved orange trees (*Citrus sinensis* (L.) Osbeck) were characterized with lesser concentration of Fe in apoplasmic fluid and cell sap from leaves which coincided with lesser expression and activity of ferric reductase, lesser content of malate and citrate as well as greater K^+:Ca^{2+} ratio in leaf blade (Martinez-Cuenca et al., 2017). However, explanations based only on activation of Fe transport to mesophyll cells within leaf blades of the studied species due to soil application of Fe-HBED seems not to be plausible, especially taking into consideration stabile Fe contents in shoots of the chlorotic-prone species. The most probable explanation of this issue is interaction between Fe and Mn, as linkage between these elements was presented to affect chlorosis (Somers and Shive, 1942). Regarding the soils used in this study, considerable differences in Fe:Mn ratio were observed (> 50.0 in Podzol and 4.7 in Rendzina). Moreover, data pertaining to Fe:Mn ratio in the plants showed that the chlorosis-susceptible species had high root Fe:Mn ratio on Podzol and significantly lower on Rendzina, whereas in the chlorosis-resistant species this parameter was less dependent of soil type. It suggests that Aa and Bo do not possess stationary nor inducible physiological adaptation to maintain Fe status by Mn sequestration. Sequestration of Mn in root vacuoles was very recently listed as a beneficial mechanism alleviating Fe-dependent chlorosis in *Arabidopsis thaliana* (L.) (Eroglu et al., 2016). It also implies that in the tested setup, diminished chlorosis resulted at least partially from competition between Mn and Fe due to quantitative increase in soil of Fe after Fe-HBED addition. It is established that Fe, Mn and other divalent ions are transported by the same transporters, e.g. IRT1 (Eroglu et al., 2016) which probably have very similar affinity to Fe and Mn (Korshunova et al., 1999). Thus, it is probable that increased Fe:Mn ratio in plants treated with 25 μmol kg^{-1} soil Fe-HBED results from increased ratio of Fe:Mn availability for transporters. This is also consistent with study on strawberry plants (*Fragaria* × *ananassa* Duch.) in which Fe-supplied non-chlorotic plants had higher Fe:Mn ratios in roots than Fe-starved chlorotic ones (1.2–2.5 times, depending on sampling date; calculated according to the data presented in Gama et al., 2016). In the same study, recovery of chlorosis by addition of 10 μM of Fe increased Fe:Mn ratio 1.7 times compared to Fe-starved plants, which supports our assumption of Fe and Mn interaction during development of Fe-dependent chlorosis. Release of Fe from its insoluble form by HBED (thus continuously increasing Fe:Mn ratio in soil) also cannot be underestimated in the experiment (Kang et al., 2019). This is however not the case in Sv and Vt; this mechanism probably does not play a major role in them, hypothetically due to adaptation similar to MTP8-dependent mechanism basing on Mn sequestration (Eroglu et al., 2016). This is also in agreement with physiognomic adaptation of Sv which acted very differentially than other species. In the case of Sv many elements (e.g. Fe, Mn and Zn) were partitioned mostly in the roots. Such mode of action can be possible due morphology and anatomy of the roots, as Sv has thick taproot with developed cortex (Koyuncu et al., 2009) suitable for storage function. Summarizing, presented data suggest that in chlorosis-susceptible species Fe:Mn ratio is controlled externally (by soil environment), whereas in chlorosis-resistant species this parameter is under internal control (e.g. genetically determined).

In conclusion, our study further supports the idea that xerothermic grasslands settled on Ca-rich soils are plant communities composed of chlorosis-susceptible and chlorosis-resistant species. The experiments indicated also that the individual Fe requirements did not correspond with individual soil pH requirements of the studied species. Moreover, Fe was showed to be an element creating micro-habitats in Rendzinas. Development of Fe-dependent chlorosis resulted in worsened functioning of photosynthetic apparatus, but alleviation of this physiological failure may lead to reduced competitiveness due to reduced growth. Calcicole plants subjected to acidic soil did not suffer from Fe deficiency but it was paid for with developmental limitations caused by other nutrients. Ultimately, physiological basis of lime chlorosis may result from inability to release Fe from its insoluble form, direct competition between soluble Fe and Mn during root acquisition or impaired allocation of Fe within leaf blade, but not from disturbances in translocation of this element from roots to shoots.

Declaration of Competing Interest

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

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Author contributions

Study conception and design were prepared by Jeremi Kołodziejek and Mateusz Wala. Material preparation was performed by Mateusz Wala. Data collection and analysis were performed by Mateusz Wala, Jeremi Kołodziejek, Janusz Mazur and Jacek Patykowski. The first draft of this manuscript was written by Mateusz Wala and Jeremi Kołodziejek. All authors commented on previous version of the manuscript, read and approved its final form.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2020.114572.

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P–1

Materiał uzupełniający

(Supplementary material)

Supplemental Table 1. Values indicating the effects of environmental conditions on the studied species.

Indicator values follows 1–9 scale proposed by Ellenberg et al. (1992). L: light requirements ranging 7–9, where 7 indicates well lit/slightly-shaded conditions (c.a. 30% of relative illumination) and 9 indicates full light conditions (> 50% of relative illumination); T: temperature requirements with value of 6 indicating moderately warm/warm conditions (characteristic for montane and sub-montane locations), K: continentality requirements ranging 3–4, where 3 indicates atlantic/subatlantic conditions and 4 indicates subatlantic conditions; F: soil moisture requirements ranging 3–4, where 3 indicates dry soils and 4 indicates dry/fresh soils; R: soil pH requirements ranging 7–9, where 7 indicates slightly acid/slightly basic soils and 9 indicate extreme basic soils originating from limestones; N: soil nitrogen requirements ranging 2–5, where 2 indicates infertile soils and 5 indicates intermediately fertile soils. *uncertain and not fully described behaviour; 0 wide amplitude or unequal behaviour in different areas with the same conditions.

Species	Ellenberg's indicator value					
	Light (L)	Temperature (T)	Continent (K)	Moisture (F)	pH (R)	Nitrogen (N)
Aa	8	6	6	4	9	3
Bo	7	6	5	*	0	3
Pg	7	0	5	3	8	3
Sv	9	6	6	4	7	5
Vt	7	6	5	3	8	2

Supplemental Table 2. Parameters derived from the OJIP transient used in this study, formulas of their calculation and definitions.

OJIP parameter	Formula	Definition
F_0 (=F_O)	F_0=F at 50µs (=F at O-step)	Fluorescence intensity at O-step (at 50 µs) (=Minimal fluorescence intensity)
F_J	F_J=F at 2ms (=F at J-step)	Fluorescence intensity at J-step (at 2 ms)
F_I	F_I=F at 60ms (=F at I-step)	Fluorescence intensity at I-step (at 60 ms)
F_M (=F_P)	F_M=F at 1s (=F at P-step)	Fluorescence intensity at P-step (at 1000 µs) (=Maximal fluorescence intensity)
F_V	$F_V=F_M–F_0$	Maximal variable fluorescence
V_J	$V_J=(F_J–F_0)/(F_M–F_0)$	Relative variable fluorescence at J-step (2 ms)
V_I	$V_I=(F_I–F_0)/(F_M–F_0)$	Relative variable fluorescence at I-step (60 ms)
F_V/F_M	-	Maximum quantum yield of primary PSII photochemistry
M_0	$M_0=TR_0/RC–ET_0/RC$	Approximated initial slope of the fluorescent transient
Area	-	Area between fluorescence curve and F_M (background subtracted)
S_M	S_M=Area/(F_M - F_0)	Standardized area above the fluorescence curve between F_0 and F_M
N	$N=S_M*M_0*(1/V_J)$	Number of Q_A redox turnovers until F_M is reached
φ_{E0}	$\varphi_{E0}=[1–(F_0/F_M)]*\psi_0$	Quantum yield for electron transport from Q_A to plastoquinone at t = 0
PI_{ABS}	$PI_{ABS}=\gamma RC/(1-\gamma RC)*\varphi_{P0}/(1-\varphi_{P0})*\psi_0/(1-\psi_0)$	Performance index of electron flux from PSII based to intersystem acceptors
ABS/RC	$ABS/RC=M_0*(1/V_J)*(1/\varphi_{P0})$	Photon flux absorbed by PSII antenna chlorophyll per RC at t = 0
TR_0/RC	$TR_0/RC=M_0*(1/V_J)$	Trapping flux leading to Q_A reduction per RC at t = 0
ET_0/RC	$ET_0/RC=M_0*(1/V_J)*\psi_0$	Electron transport flux per RC at t = 0
DI_0/RC	$DI_0/RC=(ABS/RC)–(TR_0/RC)$	Dissipated energy flux per RC at t = 0

Supplemental Table 3. Results of two-way ANOVA (*F* values) showing effects of species, treatment and their interaction on the chlorosis related parameters. ***$p < 0.001$

Parameter	*F* values and significance		
	(S) Species (*df* = 4)	(T) Treatment (*df* = 3)	S x T (*df* = 12)
Chlorophyll content	18.760***	38.680***	8.220***
IDC score	26.375***	48.333***	8.542***

Supplemental Table 4. Leaf morphometric parameters of the studied species grown in Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 µmol kg^{-1} soil Fe-HBED (r25). Values (mean ± SD) with different letters for each parameter are significantly different between the treatments at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD post-hoc test).

Species	Treatment	Lenght [cm]	Width [cm]	Area [cm^2]	Perimeter [cm]	Length:Width Ratio
Aa	p	2.45 ± 0.27c	2.08 ± 0.11b	3.81 ± 0.68c	7.61 ± 0.68c	1.18 ± 0.09b
	r	5.01 ± 0.28b	3.80 ± 0.43a	13.55 ± 2.12b	14.65 ± 1.15b	1.32 ± 0.11ab
	r5	5.39 ± 0.34b	3.65 ± 0.33a	14.16 ± 1.86ab	15.23 ± 1.04ab	1.51 ± 0.16a
	r25	6.26 ± 0.64a	4.12 ± 0.45a	18.17 ± 3.20a	17.37 ± 1.29a	1.53 ± 0.19a
Bo	p	2.92 ± 0.29b	2.13 ± 0.24a	4.87 ± 1.08b	8.87 ± 0.88b	1.38 ± 0.03a
	r	2.90 ± 0.21b	2.11 ± 0.25a	4.81 ± 0.81b	9.23 ± 1.00ab	1.39 ± 0.20a
	r5	3.76 ± 0.36a	2.63 ± 0.42a	7.71 ± 1.66a	11.19 ± 1.29a	1.45 ± 0.18a
	r25	2.90 ± 0.13b	2.22 ± 0.08a	4.84 ± 0.19b	8.94 ± 0.29b	1.30 ± 0.08a
Pg	p	3.23 ± 0.28c	2.24 ± 0.16b	5.58 ± 0.90b	9.05 ± 0.64c	1.44 ± 0.05b
	r	5.00 ± 0.18a	3.04 ± 0.13a	11.12 ± 0.95a	13.43 ± 0.58a	1.65 ± 0.03a
	r5	4.20 ± 0.35b	2.34 ± 0.18b	7.34 ± 1.10b	10.90 ± 0.97b	1.80 ± 0.08a
	r25	3.83 ± 0.28b	2.15 ± 0.19b	6.01 ± 0.68b	10.45 ± 0.53bc	1.82 ± 0.14a
Sv	p	3.96 ± 0.52c	2.60 ± 0.32c	7.62 ± 1.95c	11.53 ± 1.80b	1.54 ± 0.06a
	r	8.44 ± 0.62a	5.46 ± 0.65a	33.87 ± 6.41a	26.93 ± 4.16a	1.56 ± 0.10a
	r5	7.97 ± 0.45ab	5.20 ± 0.34a	30.39 ± 3.24a	24.62 ± 2.07a	1.53 ± 0.04a
	r25	6.61 ± 1.14b	4.06 ± 0.43b	19.64 ± 4.49b	20.27 ± 4.47a	1.62 ± 0.14a
Vt	p	1.48 ± 0.23b	1.04 ± 0.26a	1.18 ± 0.46b	4.13 ± 0.81b	1.45 ± 0.15b
	r	2.00 ± 0.17ab	1.39 ± 0.12a	2.07 ± 0.32ab	5.76 ± 0.56ab	1.45 ± 0.03b
	r5	2.54 ± 0.31a	1.39 ± 0.18a	2.60 ± 0.61a	6.95 ± 0.84a	1.86 ± 0.12a
	r25	2.18 ± 0.31a	1.39 ± 0.18a	2.22 ± 0.62ab	6.00 ± 0.87a	1.58 ± 0.03b

Supplemental Table 5. Results of two-way ANOVA (*F* values) showing effects of species, treatment and their interaction on the growth related parameters. *$p < 0.05$, ***$p < 0.001$

Factor	*F* values and significance		
	(S) Species (*df* = 4)	(T) Treatment (*df* = 3)	S x T (*df* = 12)
Root FW	71.654***	15.779***	6.155***
Root DW	92.675***	21.823***	13.713***
Shoot FW	54.100***	23.234***	5.278***
Shoot DW	40.497***	13.654***	3.937***
FW shoot:root ratio	8.847***	3.226***	0.535*
DW shoot:root ratio	10.692***	18.419***	2.453*
Leaf lenght	273.006***	90.064***	18.839***
Leaf width	227.397***	55.762***	15.564***
Leaf area	202.495***	57.482***	21.690***
Leaf perimeter	178.146***	50.220***	11.869***
Leaf lenght:width ratio	20.690***	15.650***	3.600***

Supplemental Table 6. Results of two-way ANOVA (*F* values) showing effects of species, treatment and their interaction on the OJIP-related parameters. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s. – not significant

Parameter	*F* values and significance		
	(S) Species (*df* = 4)	(T) Treatment (*df* = 3)	S x T (*df* = 12)
F_0	6.782***	2.187n.s.	0.793n.s.
F_J	6.500***	11.380***	2.060*
F_I	4.724**	11.516***	1.155n.s.
F_M	4.363**	4.640**	1.291n.s.
F_V	2.967*	2.581n.s.	2.164*
V_J	0.738n.s.	0.896n.s.	2.209*
V_I	1.399n.s.	1.451n.s.	2.439**
F_V/F_M	6.390***	1.911n.s.	1.463n.s.
M_0	11.006***	6.864***	7.954***
Area	11.845***	10.443***	1.271n.s.
S_M	22.670***	5.620**	3.750***
N	6.920***	5.810**	1.430n.s.
φ_{E0}	12.700***	13.060***	2.220*
PI_{ABS}	19.900***	11.730***	3.180***
ABS/RC	267.610***	0.890n.s.	4.030***
TR_0/RC	8.708***	1.032n.s.	5.586***
ET_0/RC	377.683***	5.462**	1.731n.s.
DI_0/RC	16.097***	4.840**	3.405***

Supplemental Table 7. Results of two-way ANOVA (*F* values) showing effects of species, treatment and their interaction on the enzymatic parameters. $^{**}P < 0.01$, $^{***}p < 0.001$

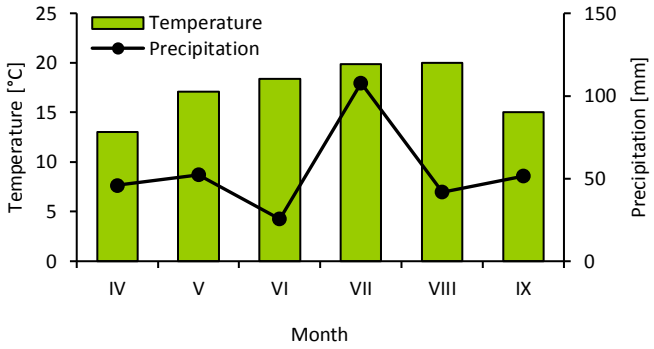
Parameter	*F* values and significance		
	(S) Species (*df* = 4)	(T) Treatment (*df* = 3)	S x T (*df* = 12)
Root SOD	96.941***	15.332***	6.142***
Root CAT	111.430***	9.673***	15.521***
Root POD	236.494***	29.716***	15.991***
Leaf SOD	372.343***	7.300***	2.580**
Leaf CAT	106.271***	12.193***	9.125***
Leaf POD	212.776***	214.431***	65.975***

Supplemental Table 8. Results of two-way ANOVA (*F* values) showing effects of species, treatment and their interaction on the elemental composition parameters. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, n.s. – not significant

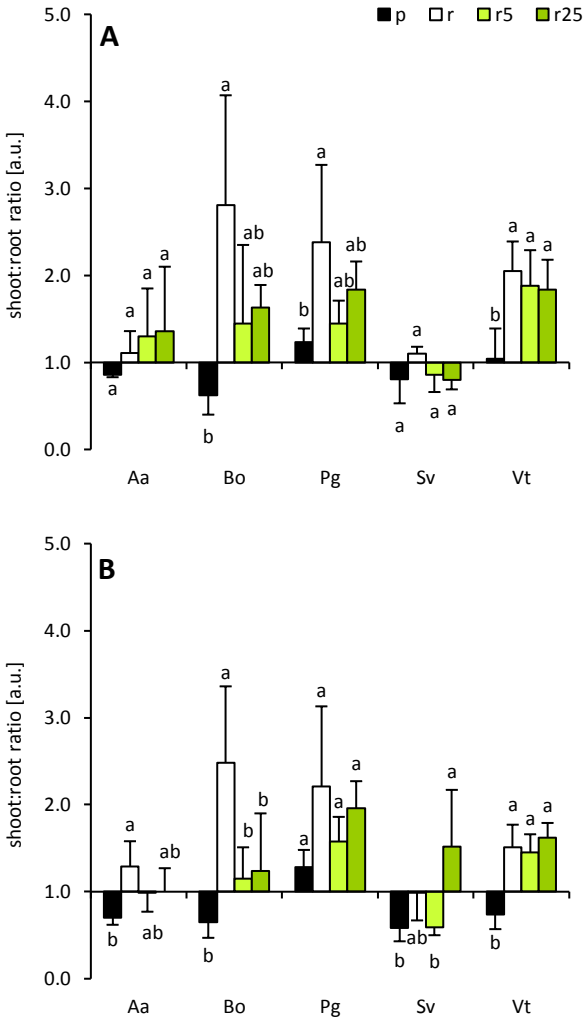
Trait	Factor	*df*	*F* values and significance					
			Ca	Mg	Fe	Mn	Zn	Cu
Root content	(S) Species	4	8.623***	108.098***	41.119***	46.052***	4.454**	5.400***
	(T) Treatment	3	5.293**	0.459n.s.	1.496n.s.	8.711***	1.059n.s.	22.356***
	S x T	12	2.929**	4.130***	6.517***	9.775***	1.363n.s.	6.936***
Shoot content	S	4	14.749***	86.726***	7.090***	89.949***	16.307***	18.475***
	T	3	0.024n.s.	1.632n.s.	0.486n.s.	17.626***	0.972n.s.	16.281***
	S x T	12	2.867**	4.603***	2.325*	13.573***	2.466*	1.318n.s.
SAP	S	4	2.179n.s.	69.517***	9.292***	71.861***	7.451***	20.343***
	T	3	1.503n.s.	17.593***	7.981***	43.289***	4.623**	13.516***
	S x T	12	4.717***	6.702***	2.732**	4.373***	2.471*	1.208n.s.

Supplemental Table 9. Results of two-way ANOVA (*F* values) showing effects of species, treatment and their interaction on the Fe:Mn ratio in roots and shoots. ***$P < 0.001$, n.s. – not significant

Fe:Mn ratio	*F* values and significance		
	(S) Species (*df* = 4)	(T) Treatment (*df* = 3)	S x T (*df* = 12)
Roots	17.169***	6.327***	7.825***
Shoots	17.517***	1.640n.s.	3.743***



Supplemental Figure 1. Mean temperature and precipitation in Łódź city during experiment (April-September 2018)



Supplemental Figure 2. Fresh weight (A) and dry weight (B) allocation measured as shoot: root ratio of the studied species grown in Podzol (p), Rendzina (w) or Rendzina with addition of 5 (r5) or 25 µmol kg^{-1} soil Fe-HBED (r25). Values (mean ± SD) with different letters for each parameter are significantly different between the treatments at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD post-hoc test).

Praca P–2

W oparciu o wyniki z pierwszego etapu badań, w drugim etapie prac skupiono się na weryfikacji hipotez odnoszących się do podatności gatunków kwasolubnych na rozwój ograniczeń zależnych od żelaza. Koncepcja badań była w swych założeniach zbliżona do pierwszego etapu prac – określono różnice międzygatunkowe odnoszące się do wymagań względem dostępności żelaza oraz zbadano kwestię złożoności etiologii niedoborów tegoż pierwiastka i ich skutków fizjologicznych. W przeciwieństwie do pierwszego etapu prac, zbadano również powiązanie biosyntezy metabolitów specjalistycznych (syn. metabolity wtórne) z żywieniem mineralnym. Doświadczenie przeprowadzono z wykorzystaniem pięciu gatunków roślin (*A. montanum*, *A. dioica*, *H. radicata*, *J. montana* i *P. arenaria*) występujących naturalnie na glebach kwaśnych w obrębie piaszczystych muraw bezwapiennych.

Zastosowany układ doświadczalny był tożsamy z tym wykorzystanym w poprzednim etapie prac; w ramach badania odtworzono wszystkie procedury związane z założeniem, utrzymaniem i terminacją doświadczenia.

Zakres testowanych hipotez (hipoteza 2, 3 i 4) był spójny z poprzednim etapem badań; zmodyfikowano jedynie pierwszą hipotezę badawczą: badane gatunki mają wyraźne preferencje względem typu gleby i są gatunkami kwasolubnymi (rosną lepiej na kwaśnej glebie bielicowej niż na zasadowej rędzinie właściwej; porównanie wariantów „p" i „r"; H1).

Wyniki wskazały, że znacząca większość badanych gatunków (za wyjątkiem *A. montanum* tolerującym oba badane typy gleb) przejawia wyraźną preferencję względem gleby o kwaśnym odczynie i rosną istotnie lepiej na glebie bielicowej niż na rędzinie właściwej. Tym samym, odrzucono hipotezę pierwszą (H1) o powszechności preferencji badanych roślin względem gleb kwaśnych. Ponadto, skala różnic między wzrostem badanych gatunków na glebie kwaśnej i zasadowej sugeruje, że rośliny te przejawiają różny stopień tolerancji względem alkalizacji środowiska. Można zaproponować, że *J. montana* i *H. radicata* gorzej znoszą wzrost odczynu gleby niż pozostałe badane gatunki, przez co prawdopodobnie szybciej zanikają w wyniku sukcesji piaszczystych muraw bezwapiennych lub ich antropogenicznego przekształcenia. Co więcej, zarówno *A. montanum*, jak i *P. arenaria*, gatunki uznawane powszechnie za rośliny zasadolubne, okazały się być roślinami o odpowiednio niezróżnicowanych wymaganiach oraz wyraźnie przesuniętych wymaganiach w kierunku gleby kwaśnej. Ponadto, ograniczenia wzrostu obserwowane na glebie zasadowej nie były wyrównywane w pełni przez suplementację żelazem, co sugeruje dodatkowe (niezależne od żelaza) źródła ograniczeń. W obrębie badanych gatunków występują zatem, podobnie jak pośród gatunków z poprzedniego etapu badań, zarówno rośliny stenotopowe, jak i eurytopowe.

Poprzez pomiar zawartości chlorofilu wykazano również znaczną różnorodność reakcji roślin na zmienną dostępność żelaza, od braku istotnych różnic (*A. montanum*), poprzez spadek zawartości warunkowany wyłącznie typem gleby (*P. arenaria*) lub wyłącznie zwiększoną dostępnością żelaza na glebie zasadowej (*H. radicata*),

do złożonej reakcji przejawiającej się chlorozą indukowaną zasadowym odczynem gleby i znoszoną poprzez zwiększenie dostępności żelaza (*A. dioica* i *J. montana*). Jedynie *A. dioica* i *J. montana* rozwinęły chlorozę mierzalną przy pomocy fenotypowej skali kategoryzacyjnej. Wyniki pozwoliły na odrzucenie hipotezy drugiej (H2) o jednorodności zdolności badanych gatunków do zachowania homeostazy w warunkach stresu alkalizacji siedliska. Ilościowe różnice we wzorcach wzrostu i zawartości chlorofilu w obrębie badanych gatunków sugerują, że zbliżone (lub niezróżnicowane) wymagania względem odczynu gleby przeciwstawiają się zróżnicowaniu podstawowych wymagań żywieniowych, których zaspokojenie pozwala na zachowanie niezachwianej biosyntezy i utrzymania chlorofilu. Preferencje względem odczynu gleb i dostępności żelaza nie są zatem ściśle ze sobą powiązane.

Podobnie jak w poprzednim etapie badań, wyniki nie pozwalały na odrzucenie hipotezy trzeciej (H3) – rośliny podatne na rozwój chlorozy nie przejawiały jej objawów po traktowaniu żelazem, co jasno wskazuje na zależną od żelaza etiologię niedoborów. Warto wspomnieć, że *H. radicata* traktowany dawką 25 µmol Fe-HBED · kg^{-1} gleby zawierał mniej chlorofilu niż rośliny uprawiane bez traktowania chelatem (porównanie pomiędzy wariantem „r" i „r25"), co może być spowodowane spadkiem zawartości magnezu (Senbayram i wsp., 2015).

Wyniki niniejszej pracy są zgodne z pierwszym etapem prac i pozwalają na spójne ujęcie problemu niedoborów żelaza (objawiających się w ciężkim przebiegu pod postacią chloroz międzynerwowych) jako ograniczeń o zróżnicowanej i często złożonej etiologii. Uszczegóławiając, chloroza zależna od żelaza, oraz wynikające z tego zmiany w funkcjonowaniu fotoukładów, związana jest z patofizjologicznym poborem manganu i cynku przez transportery o niskiej selektywności, których rolą jest również pobór żelaza. Oznacza to, że rozwój chlorozy warunkowany jest stosunkiem ilościowym między dostępnością żelaza, a manganu i cynku w glebie (oraz modyfikowany jest dostępnością miedzi). Potwierdzają to odnotowane trendy zmian zawartości badanych pierwiastków (wskazujących wprost na „paradoks chlorozy") oraz ich stosunki ilościowe. Uzyskane dane nie dają pretekstu do odrzucenia hipotezy czwartej (H4), przez co należy przyjąć, że złożona etiologia przyczynia się do wytłumaczenia zjawiska „paradoksu chlorozy zależnej od żelaza".

Znaczne różnice odnoszące się do zawartości badanych metabolitów specjalistycznych sugerują istnienie specyficznych gatunkowo adaptacji umożliwiających funkcjonowanie mechanizmów odpowiedzialnych za żywienie mineralne zgodnie z naturalnym zapotrzebowaniem. Z racji istnienia ścisłej relacji między biosyntezą, a wydzielaniem tychże metabolitów (Zwetsloot i wsp., 2018), należy uważać, że zmniejszone zawartości wybranych grup związków w korzeniach *A. dioica* i *J. montana* rosnących na glebie zasadowej (w porównaniu do roślin rosnących na glebie kwaśnej) świadczą o załamaniu funkcjonowania wspomaganego wydzielaniem metabolitów specjalistach procesu poboru żelaza. Problem ten nie występuje jednak u roślin, u których zawartość metabolitów była stale bardzo wysoka (*P. arenaria*) lub indukowana zasadowym odczynem gleby (*H. radicata*). Niniejsze dane sugerują udział metabolitów posiadających

właściwości kompleksujące oraz redukujące w procesie poboru żelaza przez rośliny wykazujące Strategię I, mimo że związki te nie są ujmowane jako kanoniczne elementy tejże strategii (Chao i Chao, 2022).

Wyniki badań zebrane na tym etapie badań jasno pokazały, że piaszczyste murawy bezwapienne, podobnie jak kserotermiczne murawy wapieniolubne, tworzone są przez gatunki o zróżnicowanych wymaganiach względem odczynu gleby i dostępności żelaza. Warto podkreślić, że w drugim etapie badań oba te wymagania nie pokrywały się zupełnie, co sugeruje oddzielność wymagań pod względem odczynu gleby i dostępności żelaza. Znaczne zwiększenie dostępności żelaza w glebie zasadowej w wyniku aplikacji Fe-HBED nie kompensuje wszystkich rodzajów ograniczeń, których doświadczają badane gatunki, co sugeruje, że dostępność żelaza nie jest dla nich jedynym czynnikiem limitującym. Co najważniejsze, niniejsze badania potwierdziły przypuszczenie o złożoności etiologii niedoborów żelaza również u gatunków kwasolubnych, co sugeruje, że może to być mechanizm uniwersalny dla większości lądowych roślin wyższych doświadczających niedoborów żelaza.

W niniejszej części pracy doktorskiej zastosowano jedną metodę doświadczalną (częściowo kontrolowane doświadczenie polowe) oraz pięć technik analitycznych (metody wagowe, laserowy skaning optyczny, spektrofluorymetria, spektrofotometria oraz spektroskopia absorpcji atomowej). W oparciu o uzyskane wyniki opublikowano autorską pracę w czasopiśmie *Journal of Plant Physiology* zatytułowaną „*The diversity of iron acquisition strategies of calcifuge plant species from dry acidic grasslands*”.

P–2

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The diversity of iron acquisition strategies of calcifuge plant species from dry acidic grasslands

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ABSTRACT

Although the calcifuge plant species existing in dry acidic grasslands are believed to be prone to iron (Fe)-dependent limitations, little is known about their susceptibility and reaction to pH-dependent Fe starvation. Therefore, the present study examines the effects of contrasting soils (acidic Podzol vs alkaline Rendzina) and Fe supplementation (Fe-HBED) on alkaline substratum (5 and 25 μmol Fe-HBED kg^{-1} soil). Five calcifuge dicotyledonous plant species (*Alyssum montanum* L., *Antennaria dioica* (L.) Gaertn., *Hypochaeris radicata* L., *Jasione montana* L. and *Potentilla arenaria* Borkh.) were tested in a pot experiment under field conditions. Chlorosis, chlorophyll content, growth and chlorophyll *a* fluorescence were measured. The elemental composition (contents of Ca, Mg, Fe, Mn, Zn and Cu) of the roots and shoots were analyzed, as well as their specialized metabolites. Two studied species (*A. dioica* d and *J. montana*) were susceptible to pH-dependent chlorosis, and this deficiency was successfully diminished by the application of Fe-HBED. Almost all the studied species (except *A. montanum*) preferred the acidic soil. Fe-HBED treatments were not sufficient for supporting the growth of *H. radicata* and *J. montana* in alkaline soil to the same degree as in acidic soil, which suggests additional non-Fe-dependent limitations. Both Fe starvation and Fe over-supplementation caused species-specific changes in chlorophyll *a* fluorescence. The disturbed Fe acquisition in the alkaline soil was not the sole source of the observed limitations, as the chlorosis-susceptible species demonstrated a complex interaction between Fe, Mn and Zn. The species resistant to lime chlorosis contained greater amounts of specialized metabolites than the susceptible plants. Our findings do not support hypothesis that all calcifuges are susceptible to Fe-dependent chlorosis: calcifuge plant species from dry acidic grasslands appear to have diverse Fe requirements and acquisition strategies.

1. Introduction

European psammophilous acidic dry grasslands (syntaxonomically classified as *Corynephorion canescentis* Klika 1934 alliance from the *Koelerio glaucae-Corynephoretea* Klika in Klika & Novak 1941 class) are associated with acidic non-calcareous sandy soils throughout Europe (Leuchner and Ellenberg, 2017). Acidic dry grasslands are composed of grasses, annual to perennial dicotyledons, mosses and lichens (Czyżewska, 1992; Leuchner and Ellenberg, 2017); however, the qualitative and quantitative occurrence of psammophilous species is not constant. The floral diversity of acidic grasslands depends on their successional stage, as well as the local climate, physical-chemical soil properties and land usage (Czyżewska, 1992; Hasse and Daniëls, 2006; Jentsch et al., 2009; Mårtensson and Olsson, 2010). Hence, depending on these factors, acidic dry grasslands are mixtures of true pioneer species, plant specialists and generalists, including various core and satellite (subordinate) species (Czyżewska, 1992; Ozinga, 2008; Assini et al., 2013). Most plant species from dry acidic grasslands are undeniable acidophiles, otherwise known as calcifuges, as calcium (Ca) availability has been linked with soil acidity (Bothe, 2015); however, some species have a rather broad tolerance to soil acidity. In general, calcifuges from dry acidic grasslands differ in their adaptation strategies, including morpho-anatomical attributes and physiological traits allowing survival under xerothermic conditions (wide spectrum of xeromorphic characteristics) and on acidic infertile soils (characteristics allowing tolerance to macronutrient scarcity and metal oversupplementation), as well as reproductive characteristics and life histories allowing successful persistence and spread (Czyżewska, 1992; Jentsch and Beyschlag, 2003). Thus, dry acidic grasslands tend to be communities characterized by a rather high diversity of terrestrial plant species.

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The physicochemical soil characteristics of temperate inland sand dunes, on which acidic dry grasslands persist, make them unsuitable substrata for luxuriant vegetation (Czyżewska, 1992; Jentsch and Beyschlag, 2003; Sparrius et al., 2013). This is due to the harsh characteristics of sandy soils originating mostly from pleistocenic sand depositions. Acidic sandy soils, particularly Podzols (Jentsch and Beyschlag, 2003; IUSS Working Group, WRB, 2015) have a remarkably loose structure, underdeveloped sorption complex and are highly acidic (Jentsch and Beyschlag, 2003; Ferro-Vázquez et al., 2020). In addition, due to their underdeveloped sorption complex, low seasonal organic matter load and podsolization (Jentsch and Beyschalg, 2003), acidic sandy soils are typically deficient in nitrogen (N), phosphorus (P), magnesium (Mg) and Ca (Abedi et al., 2013; Sparrius et al., 2013). On the other hand, Podzols are characterized by a high availability of iron (Fe), manganese (Mn) and aluminum (Al) (Abedi et al., 2013; Cornelis et al., 2018). These elements are complexed with organic matter and translocated throughout the soil profile by percolation (Ferro-Vázquez et al., 2020); this translocation results in the formation of insoluble inorganic Al- and Fe-containing compounds, most notably aluminosilicates and Fe-oxyhydroxides (ferrihydrite; Ferro-Vázquez et al., 2020) at the illuviation horizon and, sometimes, the development of hardpan (ortstein), which can halt the growth and development of plants (Lipiec et al., 2018). Additionally, the sorption of P onto Fe and Al hydroxides and oxides (Adediran et al., 2020; Lambers, 2022) further reduces P availability in decalcified sand dunes at early successional stages; however, this can be reversed by gradual accumulation of soil organic matter (Sparrius et al., 2012). The availability and speciation of Fe in Podzols (free, in crystalline and noncrystalline oxides, fixed as well as complexed by organic compounds) depend on numerous bio-geochemical processes (Adediran et al., 2020). Although considerable differences in microelemental load can be found between soil horizons, Fe, Mn and Al are highly available for plants on sandy soils when compared to other soil types (Cornelis et al., 2018; Adediran et al., 2020). Thus, plants from psammophilous grasslands encounter macronutrient starvation and supraoptimal loads of Fe, Mn and Al.

Due to the high availability of Fe in Podzols, this element should not limit plants from dry acidic grasslands. However, Podzols demonstrate a mosaic structure, with pH values ranging from 3.6 to 6.8 (Jentsch and Beyschlag, 2003; Sparrius et al., 2012), and hence considerable spatial variation in nutrient availability, including trace metals such as Fe (Bothe, 2015). This structure can be further modified by both land usage (Jentsch and Beyschlag, 2003) and various natural processes, such as the seasonal load of organic matter and the exudation, transformation and degradation of numerous organic compounds (Ferro-Vázquez et al., 2020). Hence, the considerable heterogeneity of these habitats may result in calcifuge plant species encountering Fe-dependent limitations.

Plants from dry acidic grasslands have evolved numerous structural, physiological and ecological adaptations allowing them to persist on infertile and acidic soils (Podzols) under xerothermic conditions (Jentsch and Beyschlag, 2003; Leuchner and Ellenberg, 2017). However, edaphic conditions of dry acidic grasslands probably do not drive the evolution of pH-tolerant Fe scavenging systems in calcifuges, unlike those present in alkaline xerothermic grasslands, which are characterized by Fe scarcity. Thus, calcifuges are hypothetically better adapted to Fe overload than to alkalization-dependent Fe starvation, resulting in the development of lime chlorosis (Hutchinson, 1967). However, competition for access to Fe has spurred the evolution of Fe uptake strategies in terrestrial plants. The dicotyledonous plant species investigated in this study evolved an acidification-reduction mechanism coupled with exudation of phenolic ligands, i.e. Strategy I plants (Kobayashi and Nishizawa, 2012). In this mechanism, solubilization of Fe-bearing compounds due to the release of protons (by plasmalemmic H^+-ATPases) is followed by apoplastic Fe reduction (by ferric reduction oxidases, FROs) and its uptake (by Fe importers; Zhang et al., 2019). A growing body of evidence suggests that dicotyledonous plant species enhance Fe solubilization by the release of phenolic ligands, and that this contributes to the diversification of Fe scavenging observed in coexisting plants (Tsai and Schmidt, 2017; Robe et al., 2021). Overall, Fe acquisition process is quite complex, and plants possess an array of traits facilitating its uninterrupted functioning. It may, however, vary among species and can be further modified by environment, including biotic and abiotic factors (Dai et al., 2019; Boiteau et al., 2018; Dhankhar et al., 2022). Detailed information on this topic is scarce, but it can be hypothesized that calcifuges demonstrate less efficient Fe scavenging than calcicoles, at least partially due to their lower exudation of Fe-solubilizing metabolites (Robe et al., 2021).

The mechanisms underlying Fe-dependent chlorosis are generally poorly documented, apart from studies on laboratory-grown model plant species or plants with high agronomic importance. Although some studies have examined the interaction between calcifuges and their environment, most notably regarding xeric conditions and N and P supply (Jentsch and Beyschalg, 2003; Sparrius et al., 2013; Bartelheimer and Poschlod, 2014), little is known about the Fe nutrition of these species (Jentsch and Beyschalg, 2003; Bothe, 2015). Therefore, it is unknown whether these species demonstrate broad Fe scavenging potential, or whether they are specific to soils with high Fe availability. Furthermore, it is also not known whether calcifuges, as a group, possess diverse Fe nutrition requirements, as is the case for calcicoles from alkaline grasslands (Wala et al., 2020). Thus, the following hypotheses were put forward: 1) calcifuge species from acidic dry grasslands have similar Fe demands and encounter similar Fe limitations on alkaline soils; 2) in these species, lime chlorosis is Fe-dependent and can be reversed with highly-available sources of Fe; and 3) Fe is the sole source of limitations for calcifuge species on an alkaline substratum. The tested hypotheses were intended to answer the following questions: 1) are calcifuges similar in their Fe requirements? 2) does the nature of lime chlorosis in these species lie in Fe-dependent limitations? 3) are there any other chemical-edaphic obstacles for acidophilic vegetation on alkaline soils?

2. Materials and methods

2.1. Selection of species, source of seeds, seedling establishment and growth conditions

All selected plants typically and commonly occur in dry acidic grasslands (from the *Koelerio-Corynephoretea* class; Czyżewska, 1992; Matuszkiewicz, 2022) in Poland. In addition, all plants, *viz. Alyssum montanum* L., *Antennaria dioica* (L.) Gaertn., *Hypochaeris radicata* L., *Jasione montana* L. and *Potentilla arenaria* Borkh. are dicotyledons that originated from different plant families and are representative of the studied type of plant community. They are non-annual plant species (biennial/perennial plants; Table 1) that utilize Strategy I for acquisition of Fe. Additionally, their physiognomy allows easy experimentation, as the studied species are not of small size and possess easy-to-measure organs. Ellenberg's Indicator Values (EIVs; Ellenberg et al., 1991) indicate that all are light-loving xerothermic plants adapted to very poor N availability in soil (Supplemental Table 1). In addition, all the studied plant species are tolerant to acidic sandy soils or reach their ecological optimum on such substrata (Table 1). In the case of *A. montanum* and *P. arenaria*, their high R EIVs (R = 7 and R = 8, respectively) are not reflected in data from field surveys, as these species are known to occur on sandy acidic substrata (Hegi, 1931; Czyżewska, 1992; Kołodziejek et al., 2010).

The seed material was collected from at least 20 plants of each plant species at its maturity stage in summer 2018. All were growing under optimal conditions in the Didactic-Experimental Garden of the Faculty of Biology and Environmental Protection (University of Lodz), central Poland (51° 78′ N; 19° 48' E). All maternal plants had originally been collected from seminatural areas in central Poland and then transplanted and maintained as a regular collection of the garden. All the seeds were gathered by hand and inspected under magnification;

Table 1
List of the species used in this study, their seed size, seed mass and centre of abundance. Nomenclature of studied taxa follows the Plant List (www.theplantlist.org).

Species	Abbreviation	Family	Growth form	Seed size [mm][a]	Seed mass [mg][b]	Centre of abundance of adult individuals[c]
Alyssum montanum L.	Am	Brassicaceae	Perennial herb	1.5–1.9 × 1.1–1.3	0.368 ± 0.028	sandy fields, sunny slopes, heaths, dry pine forests, river alluvials
Antennaria dioica (L.) Gaertn.	Ad	Asteraceae	Perennial herb	1.5–1.7 × 0.35–0.45	0.097 ± 0.002	heaths, sandy dunes, dry pine forests, sunny slopes, dry bogs
Hypochaeris radicata L.	Hr	Asteraceae	Perennial herb	5.0–10.0 × 0.5–0.6	0.578 ± 0.038	sandy dunes, sandy heaths, dry grasslands, dry forests
Jasione montana L.	Jm	Campanulaceae	Biennial herb	0.6–0.8 × 0.2–0.3	0.016 ± 0.001	sandy dunes, dry grasslands, heaths, dry slopes, sparse forests
Potentilla arenaria Borkh.	Pa	Rosaceae	Perennial herb	1.0–1.2 × 0.8–1.0	0.115 ± 0.013	dry grasslands, steppe meadows, sandy dunes, dry pine forests

[a] Seed size follows Bojňanský and Fargašová (2007).
[b] Seed mass measured in this study (determined by weighing 100 air-dried seeds, expressed as mean ± SD; n = 4).
[c] Centers of abundance follows Hegi (1931).

abnormal seeds were discarded from the pool. The seeds were stored for 10 days in the laboratory under dry conditions at 21 °C and then dry-stratified at 4 °C without any source of light for 10 weeks. After stratification, the seeds were weighed (four replicates of 100 seeds for each species) to obtain information about the seed material. Following this, the seedlings were established, as described by Wala et al. (2020) for calcicole plant species, using two contrasting soil types: alkaline Rendzic Leptosol (hereafter referred to as Rendzina) and Entic Podzol (referred to as Podzol). A fuller list of physical-chemical characteristics of the soil are given by Wala et al. (2020). For each species, the pool of established seedlings was >80% of the number of sown seeds.

Representative seedlings were planted, one plant per pot, in 1.5 dm^3 plastic pots on respective soil (Rendzina or Podzol) and cultivated under field conditions in the Didactic-Experimental Garden of the Faculty of Biology and Environmental Protection (University of Lodz). The garden is in a well-lit location simulating the photo-thermal conditions found on grasslands in Central Europe. The weather conditions throughout the experiment (1st April to October 1, 2019) were determined based on data from routine measurements of the Institute of Meteorology and Water Management – National Research Institute (Warszawa, Poland). The mean lowest temperature during the experiment was 10.1 °C (April), and the highest 22.2 °C (June). The total precipitation during the experiment was 210.4 mm, with a minimum in June and a maximum in September. More detailed data regarding the weather conditions are given by Wala et al. (2021). The plants were watered in accordance with their needs to maintain optimal growth conditions.

2.2. Experimental setup

The experimental setup was performed according to Wala et al. (2020); this approach allows the study of both soil pH and Fe-dependent limitations in both calcicole and calcifuge plant species. Fe-HBED was chosen as a source of highly-available chelated Fe (*N,N′*-bis(2-hydroxyphenyl)ethylenediamine-*N,N′*-diacetic acid; 7% Fe with a chelation ratio of 1:1; PPC ADOB, Poznań, Poland).

After transplantation, the seedlings were acclimatized for one week, and the plants were then divided into four groups: one grown on Podzol and not treated with Fe-HBED (p), one grown on Rendzina and not treated with Fe-HBED (r), one grown on Rendzina and treated with 5 μmol Fe-HBED kg^{-1} soil (r5) and one grown on Rendzina and treated with 25 μmol Fe-HBED kg^{-1} soil (r25). The plants were treated with 50 cm^3 of solution containing an appropriate dose of chelate (r5 and r25) or tap water (p and r). In total, five doses were administered throughout the experiment at two-week intervals. Doses of Fe-HBED were selected concerning previous studies on similar topics (Venturas et al., 2014; Wala et al., 2020, 2021) and known optimal Fe availability in artificial media used for cultivation of many other plant taxa (Asher and Edwards, 1983). The doses applied into Rendzina (alkaline soil) were intended to create an environment, where plant meets Fe starvation (r), sufficient Fe availability (r25) and slightly intermediate conditions (r5) under which plants should start to recover from Fe scarcity. This approach allows to make assumptions on source of limitations in alkaline habitats with different levels of Fe-dependent limitations (r, r5 and r25) when compared to optimal habitat type (p) for the studied calcifuge species.

The experiment started on 1st April (the day of seed sowing) and terminated on 1st October (the day of data/plant collection). All further measurements were taken as described by Wala et al. (2020) for calcicole species.

In total, 160 plants were used in the study: eight plants (from four independent replicates; n = 4) of each species (*A. montanum*, *A. dioica*, *H. radicata*, *J. montana* or *P. arenaria*) for each treatment (p, r, r5 or r25). In each group of eight plants, four were used for the morphometric, chlorophyll and elemental analyses (n = 4) and the other four were used to analyze specialized metabolites (n = 4).

2.3. Morphometric measurements of leaves and assessment of plant growth

Leaf morphometric measurements were performed using a laser area meter (CI-202; CID Bio-Science, Camas, WA, USA). All measurements were conducted on freshly-probed representative and fully developed basal leaves. The petioles were carefully cut off prior to measurement. Each leaf blade was scanned individually. In the case of the studied *P. arenaria* (palmately compound leaves), leaf width was defined as the horizontal distance (i.e. perpendicular to the leaf vertical axis) between the two most marginal points of the leaf (upper lateral left and right leaflets), and leaf length was defined as the vertical distance (i.e. parallel to the leaf vertical axis) between the two most marginal points of the leaf (distal end of the central leaflet and the lower lateral left or right leaflet), as in other studies on *Potentilla* species (Hirao et al., 2019; Liu et al., 2020). Length, width, length:width ratio, area and perimeter values were recorded for four leaves per plant and four plants per treatment.

Plant growth was determined based on the fresh (FW) and dry weights (DW) of the roots and shoots. The plant material was thoroughly washed, blotted dry with paper, weighed and dried in an oven at 60 °C for c.a. 48 h until a constant weight of samples was obtained. Then, weight allocation was calculated as shoot:root ratios (FW and DW) using dependent pairs of root and shoot weights. All measurements and calculations were determined using four plants per treatment.

2.4. Chlorosis scoring and determination of total chlorophyll content

Chlorosis indices were recorded using nondestructive measurements of chlorophyll content and iron deficiency chlorosis (IDC) scoring. Total chlorophyll content was determined using a CCM-300 portable chlorophyll content meter (Opti-Sciences Inc., Hudson, NH, USA). The

chlorophyll fluorescence ratio (F_{735}/F_{700}) was measured according to Gitelson et al. (1999), converted using preprogrammed equations and expressed in mg m^{-2}. The measurements were performed using four fully-developed leaves per plant and four plants per treatment.

The visual grading of iron chlorosis (IDC score) was conducted using a 1–5 grade scale (Wala et al., 2020) adapted from Wang et al. (2008), where the lowest grade (1) denotes no visual chlorosis and the highest grade (5) denotes severe chlorosis. For each group, four plants were used for chlorosis scoring.

2.5. Measurements of chlorophyll a fluorescence

Any polyphasic changes in chlorophyll *a* fluorescence (OJIP) transients were determined using a FluorPen FP100 handheld PAM-type fluorometer (Photon System Instruments, Drásov, Czech Republic). The leaves were preadapted to dark conditions (20 min) using standard detachable clips to initiate the opening of all reaction centers and to reduce energization-dependent fluorescence. The measurements were then taken using a standard protocol based on Strasser et al. (2004). A detailed list of the presented parameters and their calculation method is given in see Supplemental Table 2. All measurements were conducted under optimal weather conditions. The OJIP parameters were recorded on two fully-developed leaves per plant and four plants per treatment. The OJIP data were presented as the means from normalization to untreated Rendzina (r variant; as presented in Wala et al., 2020) using radar plot visualization (Berger et al., 2007).

2.6. Determination of elemental composition, elemental partitioning and elemental relations

The plant material, consisting of whole roots and shoots, used to determine FW and DW, was hot digested (140 °C) in a mixture of HNO_3 and $HClO_4$ (ratio of 4:1, v/v). Following this, the concentrations of calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) were measured in the obtained mineralizates.

All measurements were taken according to Wala et al. (2020) using a SpectrAA 300 atomic absorption spectrometer (Varian Australia Pty. Ldt., Mulgrave, Australia) with a mounted deuterium lamp for background correction. The device was operated with an air/acetylene mixture for flame generation. The device was calibrated based on the atomic absorption standards of the assayed elements (Baker Analyzed, Mallinckrodt Baker BV, Deventer, the Netherlands). The content of each element was expressed in mg g^{-1} DW or μg g^{-1} DW, depending on the results. All measurements were taken using the root and shoot mineralizates of four plants per treatment.

The transportation of elements from roots to shoots was expressed as the shoot allocation percentage (SAP; %), as reported by Wala et al. (2020), and presented using a heatmap. The heatmap was generated using the mean SAP values from four plants per treatment. The quantitative molar ratios of Fe to Mn (Fe:Mn ratio) and Fe to Zn (Fe:Zn ratio) were calculated using the elemental analysis data.

2.7. Determination of specialized metabolites and antioxidant capacity

The fresh root and leaf samples (200 mg each) were extracted with ethanol (80%, v/v; also used for further preparation of standards) on pre-chilled mortars (sample to fluid ratio of 1:10, w/v). Subsequently, the homogenates were centrifuged at 8000×*g* at 4 °C. The yielded supernatants were collected and used for further analyses. All standard curves were prepared using analytical quality agents. All measurements were taken using a SpectraMax i3 spectrophotometer (Molecular Devices, LLC, San Jose, CA, USA).

The total soluble phenolic compound content was determined spectrophotometrically (Singleton and Rossi, 1965). Each reaction mixture contained plant extract, Folin-Ciocalteu's reagent and distilled water. The samples were pre-incubated, and then 10% (w/v) Na_2CO_3 solution was added. The reaction mixtures were incubated for 60 min in darkness at 25 °C and the absorbance was measured (λ = 725 nm). The concentration of soluble phenolic compounds in the tested extracts was calculated using a standard curve for gallic acid (0–500 μmol dm^{-3} with 50 μmol dm^{-3} steps; $R^2 = 0.996$). The results were expressed as μmol GAE g^{-1} FW (gallic acid equivalents).

The total phenylpropanoid content (sum of free, esterified and glycosided cinnamic acids) was measured spectrophotometrically (Fukumoto and Mazza, 2000). Each reaction mixture was prepared using plant extract, 0.1% HCl in 95% ethanol and 2% HCl solution. Then, the absorbance was measured (λ = 320 nm). The total phenylpropanoid concentration in the tested extracts was calculated using the standard curve for caffeic acid (0–500 μmol dm^{-3} with 50 μmol dm^{-3} steps; $R^2 = 0.991$). The results were expressed as μmol CAE g^{-1} FW (caffeic acid equivalents).

The *ortho*-dihydroxyphenolic content was measured spectrophotometrically (Johnson and Schaal, 1957). Each reaction mixture was prepared using plant extract, 0.5 mol dm^{-3} HCl and Arnow's reagent. Subsequently, 0.5 mol dm^{-3} NaOH and distilled water were added, and the absorbance was measured (λ = 515 nm). The concentration of *ortho*-dihydroxyphenolics in the tested extracts was calculated using the standard curve for catechol (0–500 μmol dm^{-3} with 50 μmol dm^{-3} steps; $R^2 = 0.997$). The results were expressed as μmol CE g^{-1} FW (catechol equivalents).

The total flavonoid content was measured spectrophotometrically (Christ and Müller, 1960, as modified by Pękal and Pyrzynska, 2014). Each reaction mixture contained plant extract and $AlCl_3$ solution. The samples were incubated for 60 min in darkness at 25 °C, and the absorbance was measured (λ = 425 nm). The flavonoid concentration in the tested extracts was calculated using the standard curve for quercetin (0–500 μmol dm^{-3} with 50 μmol dm^{-3} steps; $R^2 = 0.999$). The results were expressed as μmol QE g^{-1} FW (quercetin equivalents).

The flavanol content (flavan-3-ols, catechins) was measured spectrophotometrically (Sarkar and Howarth, 1976, modified by Horszwald and Andlauer, 2011). Each reaction mixture was prepared using plant extract, 4% vanillin in 95% ethanol and 37% HCl. The reaction mixtures were incubated for 15 min in darkness at 25 °C, and the absorbance was measured (λ = 500 nm). The flavanol concentration in the tested extracts was calculated using the standard curve for (+)-catechin (0–500 μmol dm^{-3} with 50 μmol dm^{-3} steps; $R^2 = 0.995$). The results were expressed as μmol CE g^{-1} FW ((+)-catechin equivalents).

Antioxidant capacity was measured spectrophotometrically based on ferric reducing antioxidant power (FRAP; Benzie and Strain, 1996). The reaction mixtures contained working solution (Benzie and Strain, 1996) and plant extract. The absorbance (λ = 593 nm) was measured after 4 min of incubation. The antioxidant capacity of the tested extracts was calculated using a standard for Trolox (0–500 μmol dm^{-3} with 50 μmol dm^{-3} steps; $R^2 = 0.999$). The results were expressed as μmol TE g^{-1} FW (one-electron Trolox equivalents).

2.8. Statistical analysis

The experiment was set up using a randomized design. The normality of the data was checked using the Kolmogorov–Smirnov test, and the homogeneity of variance was checked using the Brown–Forsythe test (all data met the requirements for ANOVA). The effect of each treatment (p, r, r5 or r25) on each parameter in each species was analyzed using one-way ANOVA followed by Tukey's HSD *post hoc* test. Differences were considered significant at $p < 0.05$. To test the influence of species (*A. montanum*, *A. dioica*, *H. radicata*, *J. montana* and *P. arenaria*), experimental treatment (p, r, r5 or r25) and their interaction on the measured parameters, two-way ANOVA was used; differences were accepted as significant at $p < 0.05$. All statistical evaluations were conducted using Statistica v. 13.3 (Tibco Software Inc., Palo Alto, CA, USA).

3. Results

3.1. Development of chlorosis

The studied species showed four patterns of response with regard to the development of chlorosis. *A. montanum* showed no statistically significant differences in chlorophyll content depending on soil type or iron availability in the alkaline soil (pattern 1; Fig. 1). *A. dioica* and *J. montana* showed chlorosis when grown in the alkaline soil, which was associated with significantly reduced chlorophyll content (by 30% in *A. dioica* and 55% in *J. montana*; Fig. 1); in both species, physiological impairment was partially remedied when the plants were treated with Fe-HBED (pattern 2; increase in chlorophyll content by 18% in *A. dioica* and by 40% in *J. montana*, r vs r25; Fig. 1). The chlorophyll content in *H. radicata* plants did not differ significantly between soil types; however, among the plants grown on Rendzina, those on soil treated with Fe-HBED contained significantly less chlorophyll than those on untreated soil (pattern 3; 13% decrease in chlorophyll content for r vs r25; Fig. 1). In addition, the *P. arenaria* plants grown on Rendzina showed a significantly lower chlorophyll content than those on Podzol (9% loss); however, this value was not significantly altered by Fe-HBED treatment, i.e. Fe availability (pattern 4; Fig. 1). Only *A. dioica* and *J. montana* demonstrated clearly discernible phenotypic changes related to leaf chlorophyll content, measured as IDC score (Fig. 1). In both species, Fe-HBED treatment caused a significant reduction in visual chlorosis symptoms (Fig. 1). Two-way ANOVA showed that chlorophyll content and IDC score significantly depended on species, treatment and the interaction between these factors (Supplemental Table 3).

3.2. Plant performance – growth and morphometry of leaves

For four studied species (*A. dioica*, *H. radicata*, *J. montana* and *P. arenaria*), the plants grown on the alkaline substratum were smaller than those grown on the acidic soil, with a significant root DW loss of 19–80% and shoot DW loss of 57–86% depending on species (Fig. 2). The only species that did not show a clear preference for the tested soils was *A. montanum*, as no significant difference was observed between the FW or DW of the roots and shoots of the plants grown on Rendzina and those grown on Podzol (Fig. 2). Fe-HBED treatment was associated with higher FW and DW of roots and shoots of the plants grown on Rendzina (Fig. 2). However, it is worth noting that these changes were species specific; for example, 5 μmol Fe-HBED kg^{-1} soil was only beneficial for *J. montana* (72% and 69% increases in root FW and DW, respectively), while 25 μmol Fe-HBED kg^{-1} soil was beneficial for *A. montanum* (236% and 216% increases in shoot FW and DW, respectively), *A. dioica* (increase in root DW by 109%, shoot FW by 123% and shoot DW by 130%) and partially beneficial for *H. radicata* (increase in shoot FW by 68%) plants (Fig. 2). In the case of *A. dioica*, *P. arenaria* and *H. radicata*, Fe-HBED treatment caused partial DW compensation, with similar values to those for plants grown on Podzol; in addition, treatment boosted *A. montanum* growth by 134% root DW and by 112% for shoot DW compared to Podzol (Fig. 2). Fe-HBED treatment also resulted in slight but significant increases in the FW and DW of roots in *J. montana* (Fig. 2).

The type of the studied substratum (Podzol vs Rendzina) also affected weight allocation on the root:shoot axis. For example, alkaline substratum increased the shoot:root ratio from 1.11 to 1.55 (FW) and from 1.69 to 2.35 (DW) in *A. dioica*, but decreased it from 2.71 to 1.67 (FW) in *J. montana* and from 2.11 to 0.96 (FW) and 3.27 to 1.69 (DW) in *P. arenaria* (Supplemental Fig. 1). This allocation of biomass did not differ significantly between Podzol- and Rendzina-grown plants in the case of *A. montanum* and of *H. radicata* plants (Supplemental Fig. 1). The application of Fe-HBED caused a significant decrease in the shoot:root ratio only in *A. montanum*: the value fell from 4.55 to 2.68 for DW (comparing the r and r5 variants) and from 5.04 to 2.60/3.39 for FW (comparing the r and r5/r25 variants; Supplemental Fig. 1. The results of the two-way ANOVA indicated that all the measured growth parameters depended significantly on species, treatment and the interaction between species and treatment (Supplemental Table 4). Significant differences in leaf size were found between tested substrata with regard to species (Supplemental Fig. 2). Comparing plants grown on Podzol and those grown on Rendzina, the leaves of *A. montanum* and *P. arenaria* plants did not differ significantly for any measured morphometric parameter (Supplemental Table 5). With some exceptions (e.g., leaf width in *J. montana*), all the other species (*A. dioica*, *H. radicata* and *J. montana*) showed a significant reduction in leaf length (22–51%, depending on species), width (7% for *A. dioica* and 27% for *H. radicata*), area (10–40%, depending on species) and perimeter (8–42%, depending on species) when the plants were grown on the alkaline soil (Supplemental Table 5). Interestingly, the observed changes in leaf length and width were not proportional in *A. dioica* and *J. montana*: the Rendzina-grown plants showed significantly lower smaller length:width values than those grown on Podzol (16% decrease for *A. dioica* and 45% for *J. montana*; Supplemental Table 5). The changes caused by the application of Fe-HBED were dependent on both dose used and species (Supplemental Table 5). The leaves of r5 and r25 variants were larger than those of the r variant: they demonstrated significantly greater length (up to 52% in *A. montanum*, 31% in *A. dioica*, and 63% in *J. montana*), width (up to 46% in *A. montanum* and 18% in *A. dioica*), area (up to 116% in *A. montanum*, 44% in *A. dioica*, and 58% in *H. radicata*) and perimeter (up to 48% in *A. montanum*, 27% in *A. dioica* and 57% in *J. montana*) (Supplemental Table 5). Fe-HBED treatment resulted in significantly greater leaf length:width ratio in only *A. dioica* (increase by 11%) and *J. montana* (increase by 73%; Supplemental Table 5); no such change was observed in the other studied species (*A. montanum*, *H. radicata*, and *P. arenaria*) (Supplemental Table 5). All

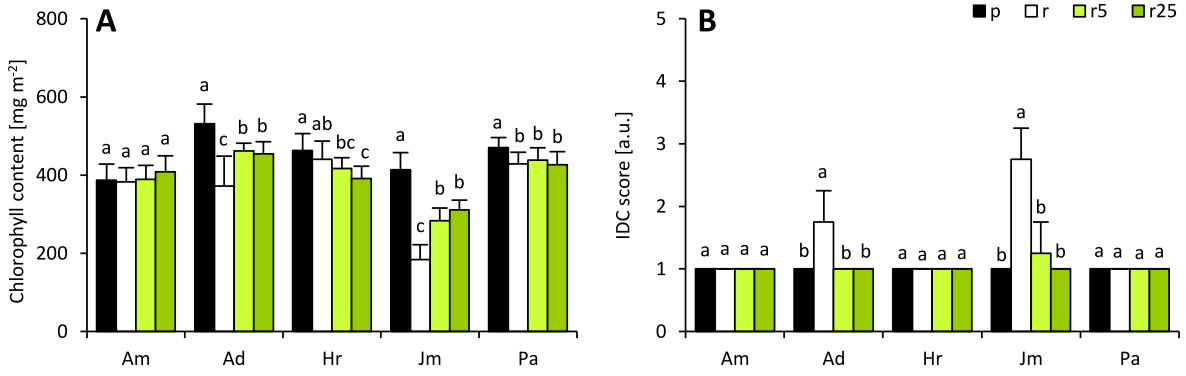

Fig. 1. Chlorosis measured as total chlorophyll content (A) and estimated using IDC scoring (B) in the leaves of the studied species grown in Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 μmol kg^{-1} soil Fe-HBED (r25). Values (mean ± SD) with the same letters for each parameter are not significantly different between the treatments within a given species at $p < 0.05$ (n = 16 for A and n = 4 for B; ANOVA with Tukey's HSD *post hoc* test).

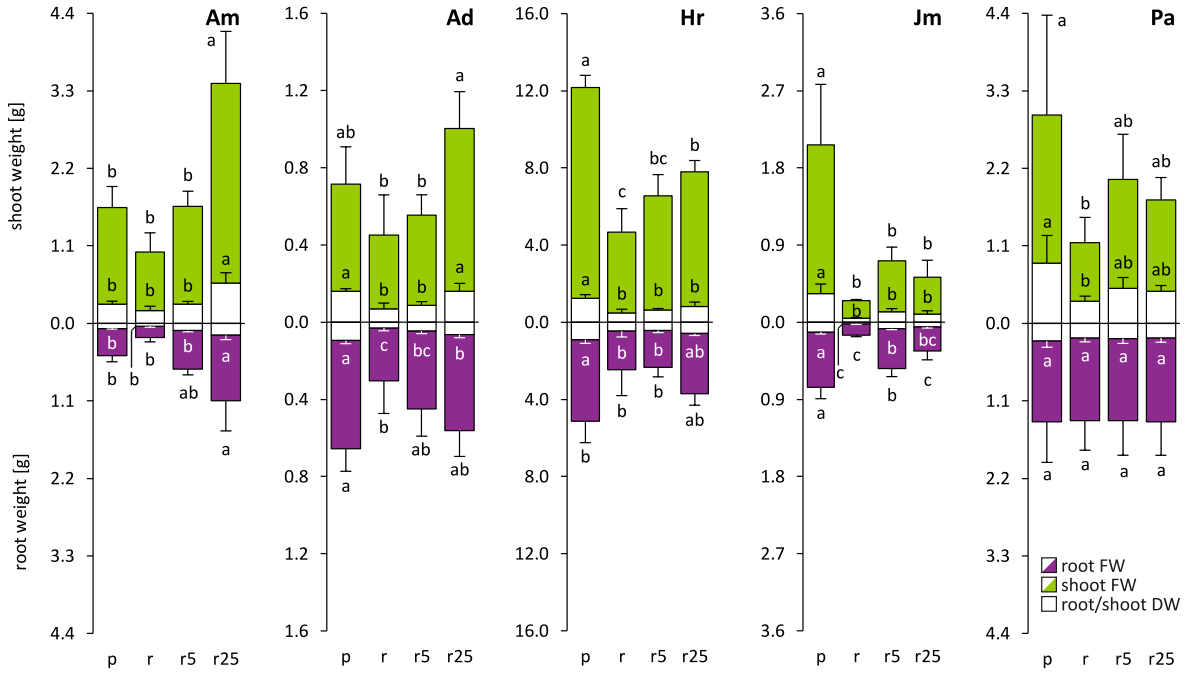

Fig. 2. The effect of Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 µmol kg^{-1} soil Fe-HBED (r25) on growth measured as fresh weights (FWs) and dry weights (DWs) of roots and shoots of the studied species. Values (mean ± SD) with the same letters for each parameter are not significantly different between the treatments within a given species at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD *post hoc* test). Whole bar – shoot/root FW, transparent bars – shoot/root DW.

the measured leaf growth and morphometrical traits depended significantly on species, treatment and the interaction between these factors (two-way ANOVA; Supplemental Table 4).

3.3. Fluorescence of chlorophyll a

Substratum type and Fe-HBED treatment differentially affected the fluorescence of chlorophyll *a*. The least differences were observed in *A. montanum*, in which the studied substrata did not trigger any major changes in the studied parameters (Fig. 3). The only significant changes in chlorophyll *a* fluorescence in this species were observed due to the application of 25 µmol Fe-HBED kg^{-1} soil (increase in F_V/F_M and decrease in DI_0/RC; Fig. 3). In the case of *A. dioica*, only the value of V_J was significantly affected by the soil type, and significant changes (increase in ET_0/RC and decrease in V_J) were only noted after treatment with 25 µmol Fe-HBED kg^{-1} soil (Fig. 3). In *H. radicata*, the only significant changes in chlorophyll *a* fluorescence were observed following Fe-HBED application. Both studied Fe-HBED doses (5 and 25 µmol kg^{-1} soil) caused significant increases in ABS/RC, TR_0/RC, ET_0/RC and DI_0/RC values and significant decreases in F_V/F_M and PI_{ABS} values (Fig. 3). The most complex changes were recorded in *J. montana*. The plants grown on the alkaline soil showed significantly lower values of F_P, F_V, ET_0/RC, φ_{E0} and PI_{ABS}, as well as significantly higher values of N, S_M, M_0, V_J and V_I than the respective ones from the acidic soil (Fig. 3). Dose-dependent significant increases in N, F_0, F_J, F_I, F_P, F_V, ABS/RC, TR_0/RC, ET_0/RC and DI_0/RC values and significant decreases in S_M, Area and PI_{ABS} values were observed as a result of Fe-HBED application (comparing Fe-HBED-treated and Fe-HBED-untreated Rendzina-grown plants; Fig. 3). In the case of *P. arenaria*, a significant reduction in F_V/F_M and PI_{ABS} and a significant increase in F_0, M_0, ABS/RC, TR_0/RC, ET_0/RC and DI_0/RC values were observed in the Rendzina-grown plants compared to those grown on Podzol (Fig. 3). Fe-HBED treatments did not alter the measured parameters compared to the plants on untreated Rendzina (Fig. 3).

The two-way ANOVA indicated that all the measured OJIP traits depended significantly on species and treatment type (Supplemental Table 5). Only N values depended significantly on the interaction between the studied factors (Supplemental Table 6).

3.4. Mineral composition, element partitioning and relation of iron to manganese and zinc

The experimental conditions affected the studied species in different ways. Regarding root composition, the plants grown on Rendzina did not contain significantly more Ca and Cu than those grown on Podzol; however, a 134% gain in Ca was observed for *P. arenaria* (Table 2). The greatest differences were observed for the other assayed elements. The roots of plants grown on Rendzina contained significantly more (*J. montana*; increase by 90%) or less (*A. montanum*, *A. dioica*, *H. radicata*; 19%, 24% and 26% loss, respectively) Mg, more Fe (*A. dioica* and *J. montana*; increase by 136% and 121%, respectively), more Mn (*A. dioica* and *P. arenaria*; increase by 518% and 121%, respectively) and more Zn (*A. dioica* and *J. montana*; increase by 518% and 70%, respectively) compared to the Podzol plants (Table 2). Fe-HBED treatments also induced dose-dependent changes in the content of the studied elements. The Fe-HBED treatments caused a significant increase in Ca content in *A. montanum* (by 187% in plants treated with 5 µmol kg^{-1} soil) and decrease in *P. arenaria* (by 42 and 56% in plants treated with 5 and 25 µmol kg^{-1} soil, respectively). It also caused an increase in Mg content in *A. montanum* (by 22% in plants treated with 5 µmol kg^{-1} soil) and decrease in *A. dioica* (by 28% in plants treated with 25 µmol kg^{-1} soil), as well as an increase in Fe content in *A. montanum* (by 276% in plants treated with 5 µmol kg^{-1} soil) and decrease in *A. dioica* (by 34% in plants treated with 25 µmol kg^{-1} soil) and *P. arenaria* (by 60 and 63% in plants treated with 5 and 25 µmol kg^{-1} soil, respectively). Treatment also resulted in increased Mn content in *A. montanum* (by 264% in plants treated with 5 µmol kg^{-1} soil) and decreased content in *A. dioica* (by 44% and 71% in plants treated with 5 and 25 µmol kg^{-1} soil, respectively) and *J. montana* and *P. arenaria* (by 76% and 47%, respectively, in plants treated with 25 µmol kg^{-1} soil). It also decreased Zn content in *A. dioica* and *J. montana* (by 18% and 35%, respectively, in plants treated with 25 µmol kg^{-1} soil). Finally, it increased the Cu content in

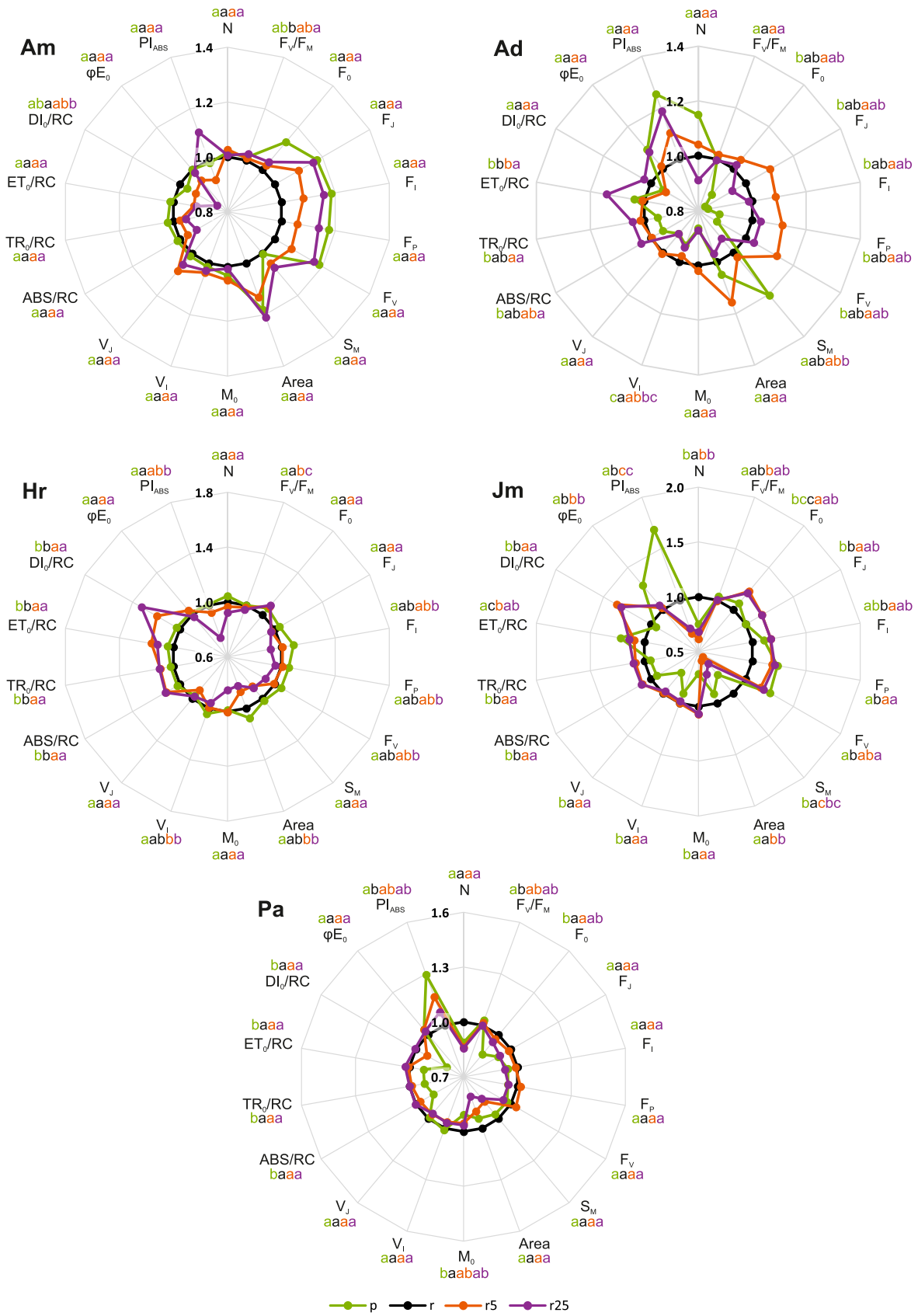

Fig. 3. Relative changes of chlorophyll *a* fluorescence parameters (OJIP) in leaves of the studied species grown on Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 µmol kg^{-1} soil Fe-HBED (r25). Relative values (means) with the same letters for each parameter are not significantly different between the treatments within a given species at $p < 0.05$ (n = 8; ANOVA with Tukey's HSD *post hoc* test). The earlier letter indicates the higher value of parameter. The color of letter-based statistical indicators corresponds with experimental groups as indicated in the legend. A description of the studied parameters is given in Supplemental Table 2. The references to the colors in this figure are given in the web version of the article.

Table 2
Elemental composition of roots and shoots of the studied species grown in Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 µmol kg^{-1} soil Fe-HBED (r25). Values (mean ± SD) with different letters for each parameter are significantly different between the treatments at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD *post hoc* test).

Treatment		Ca [mg g^{-1} DW]	Mg [mg g^{-1} DW]	Fe [mg g^{-1} DW]	Mn [mg g^{-1} DW]	Zn [mg g^{-1} DW]	Cu [µg g^{-1} DW]
		Roots					
Am	p	3.714 ± 1.206b	2.266 ± 0.109a	0.418 ± 0.184b	0.019 ± 0.003b	0.481 ± 0.092a	4.712 ± 1.266b
	r	4.365 ± 0.488b	1.836 ± 0.312b	0.445 ± 0.098b	0.014 ± 0.004b	0.422 ± 0.077a	4.533 ± 0.935b
	r5	12.545 ± 5.222a	2.236 ± 0.138a	1.673 ± 0.742a	0.051 ± 0.018a	0.697 ± 0.217a	15.594 ± 7.639a
	r25	8.615 ± 5.153ab	2.115 ± 0.115ab	1.055 ± 0.686ab	0.039 ± 0.020ab	0.597 ± 0.201a	10.755 ± 2.204ab
Ad	p	4.793 ± 0.499a	2.931 ± 0.277a	0.371 ± 0.050c	0.011 ± 0.001c	1.041 ± 0.036b	7.811 ± 0.972ab
	r	9.736 ± 5.406a	2.218 ± 0.288b	0.875 ± 0.177a	0.068 ± 0.025a	1.376 ± 0.102a	11.163 ± 3.488a
	r5	5.957 ± 0.880a	1.828 ± 0.176bc	0.675 ± 0.150ab	0.038 ± 0.004b	1.291 ± 0.120ab	5.672 ± 0.130b
	r25	5.212 ± 0.996a	1.602 ± 0.131c	0.575 ± 0.144bc	0.020 ± 0.007bc	1.125 ± 0.174b	4.399 ± 1.728b
Hr	p	2.719 ± 0.147a	1.690 ± 0.164a	0.145 ± 0.015b	0.006 ± 0.001a	0.143 ± 0.011a	4.792 ± 0.689a
	r	6.014 ± 2.537a	1.259 ± 0.258b	0.550 ± 0.337ab	0.017 ± 0.010a	0.175 ± 0.090a	5.296 ± 1.900a
	r5	3.541 ± 0.502a	1.216 ± 0.153b	0.486 ± 0.060ab	0.014 ± 0.003a	0.140 ± 0.007a	5.296 ± 0.611a
	r25	5.737 ± 1.791a	1.474 ± 0.122ab	0.762 ± 0.375a	0.020 ± 0.008a	0.201 ± 0.035a	5.296 ± 1.363a
Jm	p	1.836 ± 0.242a	2.554 ± 0.353b	0.406 ± 0.084b	0.023 ± 0.005ab	0.422 ± 0.043c	3.997 ± 0.820a
	r	4.919 ± 0.779a	4.854 ± 1.615a	0.896 ± 0.238a	0.029 ± 0.005a	0.713 ± 0.053a	5.183 ± 1.694a
	r5	12.366 ± 12.675a	3.243 ± 0.691ab	0.854 ± 0.175a	0.026 ± 0.010ab	0.597 ± 0.134ab	3.660 ± 1.519a
	r25	7.330 ± 0.953a	3.187 ± 0.826ab	1.001 ± 0.189a	0.007 ± 0.014b	0.466 ± 0.058bc	4.323 ± 1.361a
Pa	p	8.541 ± 0.206b	4.321 ± 0.196b	0.851 ± 0.117ab	0.024 ± 0.002c	0.741 ± 0.173b	10.048 ± 0.784ab
	r	20.018 ± 4.644a	5.168 ± 0.247ab	1.877 ± 0.951a	0.077 ± 0.019a	1.016 ± 0.019ab	11.041 ± 1.831a
	r5	11.664 ± 3.608b	5.760 ± 0.297a	0.743 ± 0.186b	0.062 ± 0.011ab	1.102 ± 0.093ab	7.501 ± 0.936b
	r25	8.863 ± 1.047b	5.324 ± 0.762a	0.689 ± 0.184b	0.041 ± 0.013bc	1.288 ± 0.255a	8.929 ± 1.525ab
		Shoots					
Am	p	23.082 ± 2.077ab	4.670 ± 0.628a	0.084 ± 0.017a	0.038 ± 0.012a	0.178 ± 0.019b	3.993 ± 1.037a
	r	19.397 ± 3.551b	3.553 ± 0.166b	0.160 ± 0.092a	0.017 ± 0.003b	0.204 ± 0.026b	2.088 ± 0.220b
	r5	21.679 ± 1.935ab	3.805 ± 0.134b	0.134 ± 0.015a	0.022 ± 0.002b	0.281 ± 0.050a	5.107 ± 1.116a
	r25	25.004 ± 1.221a	3.416 ± 0.197b	0.137 ± 0.028a	0.017 ± 0.002b	0.195 ± 0.041b	3.897 ± 0.557a
Ad	p	10.313 ± 1.273b	2.453 ± 0.095a	0.083 ± 0.011b	0.021 ± 0.001c	0.225 ± 0.029b	4.340 ± 0.480a
	r	12.558 ± 1.572a	2.005 ± 0.249b	0.133 ± 0.019a	0.112 ± 0.007a	0.302 ± 0.030a	6.198 ± 1.293a
	r5	8.614 ± 0.186bc	1.487 ± 0.087c	0.094 ± 0.019ab	0.084 ± 0.005b	0.259 ± 0.027ab	6.280 ± 0.764a
	r25	6.869 ± 0.494c	1.299 ± 0.063c	0.111 ± 0.032ab	0.024 ± 0.004c	0.283 ± 0.030ab	5.397 ± 2.494a
Hr	p	27.094 ± 3.310ab	4.548 ± 1.129a	0.095 ± 0.023b	0.037 ± 0.006a	0.228 ± 0.073a	6.041 ± 0.979a
	r	29.722 ± 6.010a	3.765 ± 0.527ab	0.150 ± 0.026ab	0.027 ± 0.003ab	0.309 ± 0.034a	6.925 ± 1.330a
	r5	19.651 ± 1.705b	3.415 ± 0.531ab	0.160 ± 0.022a	0.023 ± 0.004b	0.279 ± 0.065a	5.863 ± 0.944a
	r25	20.710 ± 4.084b	2.624 ± 0.456b	0.133 ± 0.043ab	0.023 ± 0.006b	0.238 ± 0.068a	5.753 ± 1.011a
Jm	p	13.090 ± 2.686a	7.205 ± 1.207a	0.127 ± 0.021b	0.069 ± 0.017ab	0.299 ± 0.040ab	6.958 ± 0.916a
	r	13.232 ± 2.703a	2.921 ± 0.263b	0.233 ± 0.040a	0.054 ± 0.005ab	0.359 ± 0.019ab	3.768 ± 0.365b
	r5	15.598 ± 1.808a	3.216 ± 0.890b	0.222 ± 0.066a	0.070 ± 0.008a	0.393 ± 0.084a	3.244 ± 0.311bc
	r25	17.142 ± 2.333a	3.321 ± 0.128b	0.198 ± 0.039ab	0.046 ± 0.011b	0.275 ± 0.034b	2.371 ± 0.013c
Pa	p	16.502 ± 2.109b	4.572 ± 0.400a	0.127 ± 0.038a	0.053 ± 0.013bc	0.123 ± 0.019a	4.951 ± 0.925a
	r	21.688 ± 1.744a	3.873 ± 0.477a	0.158 ± 0.073a	0.079 ± 0.010a	0.127 ± 0.019a	3.642 ± 0.406b
	r5	22.524 ± 2.105a	4.195 ± 0.341a	0.115 ± 0.060a	0.071 ± 0.010ab	0.134 ± 0.006a	3.500 ± 0.355b
	r25	20.160 ± 0.710ab	4.020 ± 0.188a	0.123 ± 0.042a	0.035 ± 0.008c	0.137 ± 0.012a	3.991 ± 0.223ab

A. montanum (by 244% in plants treated with 5 µmol kg^{-1} soil) while decreasing it in *A. dioica* (by 49% and 61% in plants treated with 5 and 25 µmol kg^{-1} soil, respectively) and *P. arenaria* (by 32% in plants treated with 5 µmol kg^{-1} soil; Table 2).

Some changes were also observed in the shoots. *A. dioica* and *P. arenaria* plants grown on Rendzina contained significantly more Ca than the corresponding plants grown on Podzol (increase by 22% and 31%, respectively; Table 2). In addition, some species contained significantly less Mg when grown on the alkaline soil than on the acid soil: the contents in *A. montanum*, *A. dioica* and *J. montana* decreased by 24%, 18% and 59%, respectively (Table 2). Surprisingly, two chlorosis-prone species (*A. dioica* and *J. montana*) contained more Fe when grown on Rendzina compared to Podzol (increase by 60% and 83%, respectively; Table 2). In addition, cultivation on Rendzina resulted in significant changes in shoot Mn content (433% and 49% gain in *A. dioica* and *P. arenaria*; respectively; 55% loss in *A. montanum*) compared to Podzol (Table 2). Significant differences in Zn content between the plants grown on Rendzina and Podzol were only observed for *A. dioica*

(increase by 34% on Rendzina; Table 2). The alkaline substratum caused a significant decrease in Cu content in *A. montanum*, *J. montana* and *P. arenaria* when compared to the acidic substratum (48%, 45% and 26% loss, respectively; Table 2). In addition, chelate treatment significantly changed the shoot Ca content (29% increase in *A. montanum* treated with 25 μmol Fe-HBED kg^{-1} soil; 31% and 45% decreases in *A. dioica*, and 34 and 30% decreases in *H. radicata*, following treatment with 5 and 25 μmol Fe-HBED kg^{-1} soil, respectively) (Table 2). The Mg content did not change substantially with Fe-HBED treatment, except for *A. dioica*, where a significant decrease in Mg content (26% and 35%) was observed (Table 2). Fe-HBED chelate application did not significantly affect the shoot Fe content. However, it significantly reduced shoot Mn content in *A. dioica* (by 25% and 79% due to the treatment with 5 and 25 μmol Fe-HBED kg^{-1} soil, respectively) and in *P. arenaria* (by 56% due to the treatment with 25 μmol Fe-HBED kg^{-1} soil; Table 2). Fe-HBED treatment also triggered significant changes in Zn content (38% increase in *A. montanum* following 5 μmol Fe-HBED kg^{-1} soil; Table 2) as well as in Cu content (145% and 86% increases in *A. montanum* after 5 and 25 μmol Fe-HBED kg^{-1} soil; 37% decrease in *J. montana*) (Table 2).

The content of the analyzed elements depended significantly on species, treatment and the interaction between these two factors, except root Mg and Ca content (species), shoot Ca content (treatment) and shoot Fe content (interaction between species and treatment; two-way ANOVA; Supplemental Table 7).

The shoot allocation percentage (SAP) of the assayed elements was species-specific and was differentially affected by the Fe-HBED treatments. Rendzina-grown *H. radicata* and *P. arenaria* plants showed significantly lower SAP values of Ca than the Podzol-grown plants (9% and 27% loss, respectively; Fig. 4). Mg translocation was significantly lower in the Rendzina-grown *J. montana* and *P. arenaria* plants than in the Podzol-grown ones (35% and 30% loss), while the opposite was observed in the case of *A. dioica* (increase by 16%; Fig. 4). Substratum-dependent shifts in Fe SAP values were observed solely in *H. radicata*; this species translocated less Fe from roots to shoots when grown on the alkaline than on the acidic soil (45% loss; Fig. 4). The SAP values of Zn and Cu were affected significantly by the studied substrata only in *P. arenaria* (50 and 42% loss, respectively; Fig. 4). Fe-HBED did not trigger any significant changes in SAP values in *H. radicata* and *J. montana* (Fig. 4), but it significantly affected Ca, Mg, Fe, Mn and Cu SAP values in *A. montanum*, Cu SAP values in *A. dioica* and Ca SAP value in *P. arenaria* (Fig. 4).

The SAP values depended significantly on species, treatment and the interaction between these two factors. However, the Zn SAP values did not depend significantly on treatment (two-way ANOVA; Supplemental Table 7).

Clear differences in Fe:Mn ratio were found between the studied species. In roots, this parameter was significantly higher in *A. montanum* and *H. radicata* grown on Rendzina compared to the plants grown on Podzol: 56% and 37% increases, respectively. The opposite trend was observed in *A. dioica*, which demonstrated a 60% decrease (Fig. 5). Fe-HBED application only significantly affected this parameter in *A. dioica*, where an increase of 120% was noted following 25 μmol Fe-HBED kg^{-1} soil treatment; however, a similar trend was also observed in *J. montana* (Fig. 5). Similar changes were also observed in shoots. The *A. montanum* and *J. montana* plants from the alkaline substratum had significantly higher shoot Fe:Mn ratios than the respective plants from the acidic soil (increases of 296% and 128%, respectively; Fig. 5). The opposite situation was observed in *A. dioica* (70% loss; Fig. 5). Fe-HBED treatment only significantly increased the Fe:Mn ratio in the *A. dioica* shoots (increase of 301%; Fig. 5). The Fe:Mn ratios in both roots and shoots depended on species, treatment and the interaction between species and treatment (two-way ANOVA; Supplemental Table 8). *A. dioica* and *H. radicata* grown on Rendzina showed significantly higher root Fe:Zn ratios than the respective plants from Podzol (increase by 79% and 201%, respectively; Fig. 5). The Fe-HBED treatment significantly increased the Fe:Zn ratio in the roots of *A. montanum* (increased by 121% following 5 μmol Fe-HBED kg^{-1} soil application) and *J. montana* (increased by 75% following 25 μmol Fe-HBED kg^{-1} soil application), but decreased it in *P. arenaria* (both studied doses; decreased by 71–79%; Fig. 5). Regarding the effects of soil type, the only differences in shoot Fe:Zn ratio were observed in *J. montana*; in this case, the Rendzina plants had significantly higher Fe:Zn ratios than the Podzol plants (an increase of 410%; Fig. 5). Chelate treatment did not significantly alter the Fe:Zn ratios in the shoots of the Rendzina plants (Fig. 5). Briefly, while the root Fe:Zn ratio depended significantly on species, treatment and their interaction, the shoot Fe:Zn ratio was significantly dependent only on species (Supplemental Table 8).

3.5. Specialized metabolites and antioxidant power

The studied species demonstrated three different reaction patterns regarding root phenolic compound content. *A. montanum* and *A. dioica* contained significantly lower amounts of phenolics in the roots when grown on the alkaline soil (26% and 60% loss, respectively), while the opposite was observed in *H. radicata* (55% increase; Fig. 6). On the other hand, no significant soil-dependent changes were observed in *J. montana* and *P. arenaria*, but the lowest soluble phenolic compound content in roots was observed in *J. montana* and the highest in *P. arenaria* (Fig. 6). Fe-HBED treatment significantly altered the content of phenolic compounds compared to the untreated Rendzina-grown plants, but only in *H. radicata* treated with 5 μmol Fe-HBED kg^{-1} soil (35% loss; Fig. 6). Some plants grown in Rendzina contained significantly lower phenylpropanoids (37% in *A. montanum* and 63% in *A. dioica*) and *ortho*-dihydroxyphenolics (53% in *A. montanum* and 81% in *A. dioica*) than the respective plants grown in Podzol (Fig. 6); however, the soil type did not appear to alter metabolite biosynthesis for *H. radicata* or *J. montana*

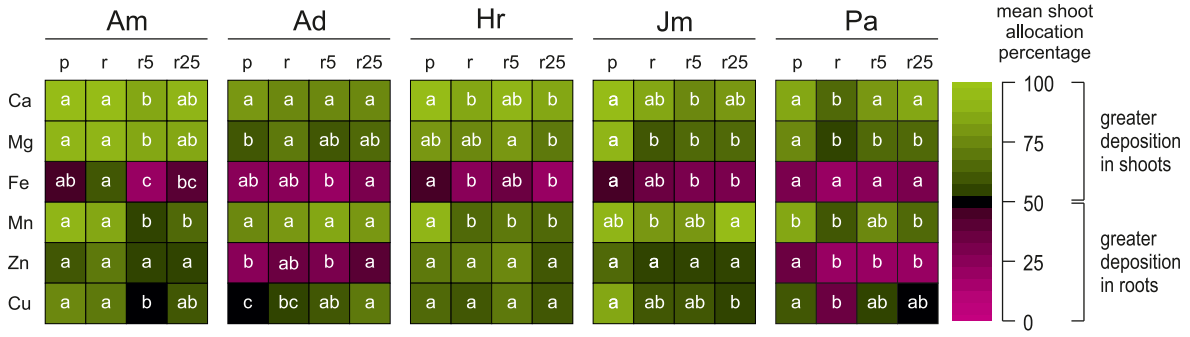

Fig. 4. Heatmap representing shoot allocation percentage (SAP) of the assayed elements (Ca, Mg, Fe, Mn, Zn and Cu) in the studied species grown on Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 μmol kg^{-1} soil Fe-HBED (r25). Values (means) with the same letters for each parameter are not significantly different between the treatments within a given species at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD *post hoc* test). An earlier letter indicates a higher value. The references to the colors in this figure are given in the web version of the article.

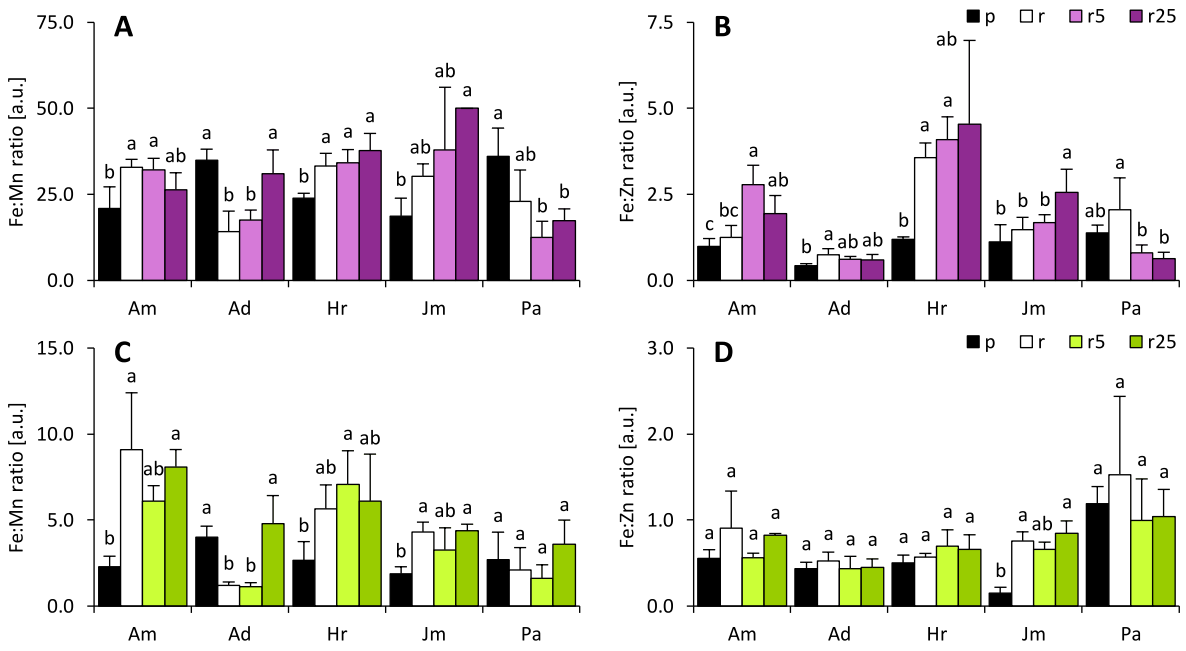

Fig. 5. Ratios of iron to manganese (Fe:Mn ratio) and iron to zinc (Fe:Zn ratio) in roots (A, B) and shoots (C, D) of the studied species grown on Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 μmol kg^{-1} soil Fe-HBED (r25). Values (mean ± SD) with the same letters for each parameter are not significantly different between the treatments within a given species at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD *post hoc* test).

(Fig. 6). Fe-HBED treatment had some effect on the phenylpropanoid or *ortho*-dihydroxyphenolic contents: it significantly increased the content of *ortho*-dihydroxyphenolics in *P. arenaria* (59% increase in the r25 variant) and decreased the content of phenylpropanoids in *P. arenaria* (24% decrease in the r5 variant; Fig. 6). The Rendzina-grown plants (*A. montanum*, *A. dioica* and *J. montana*) contained significantly less flavonoids than the Podzol-grown individuals (38%, 41% and 44%, respectively; Fig. 6); however, similar amounts of these compounds were found in *H. radicata* and *P. arenaria* grown on Podzol and Rendzina (Fig. 6). Fe-HBED treatment only significantly affected flavonoid content in *A. montanum* (32% loss in plants subjected to 25 μmol Fe-HBED kg^{-1}; Fig. 6). A very similar pattern was observed in the case of flavanols, but with exceptions for *J. montana* and *P. arenaria* (Fig. 6). The antioxidant capacity of the root extracts was not altered by soil type or chelate treatment; however, *A. dioica* demonstrated significantly higher antioxidant capacity when grown on Podzol (186% higher than Rendzina-grown plants; Fig. 6). All the tested parameters depended on the species, treatment and interaction of these two factors (Supplemental Table 9).

Some changes were also observed in the leaves. Significantly lower (15% and 44% loss) soluble phenolic compound content was noted in Rendzina-grown *A. montanum* and *H. radicata* than the respective Podzol-grown plants; these changes were reversible by Fe-HBED in *A. montanum* but not in *H. radicata*. The opposite was observed for *J. montana* (Fig. 6). No significant differences in soluble phenolic content were noted in *A. dioica* or *P. arenaria* (Fig. 6). Significantly lower phenylpropanoid contents were noted in *A. montanum* and *A. dioica* grown on Rendzina (37 and 46% loss, respectively; this change was reversible by Fe-HBED in *A. montanum*) compared to those on Podzol. A similar but insignificant reaction was observed in *H. radicata* (Fig. 6). The soil type and chelate treatment did not significantly influence the phenylpropanoid content in the leaves of *P. arenaria*, and an unclear response was observed in *J. montana* (Fig. 6). The soil type significantly affected the contents of *ortho*-dihydroxyphenolics only in *H. radicata* (59% loss), while the application of Fe-HBED affected it only in *A. montanum* (78% increase; r vs r25 comparison; Fig. 6). The alkaline soil negatively affected flavonoid content in *A. montanum* (34% loss) and *A. dioica* (50% loss) when compared to the Podzol-grown plants (Fig. 6). Additionally, *J. montana* plants subjected to 5 μmol Fe-HBED kg^{-1} soil showed a significantly lower flavonoid content (24% loss) than the respective Rendzina-grown plants (Fig. 6). Neither soil type not chelate treatment had any significant effect on leaf flavanol content (Fig. 6). The leaves of Rendzina-grown *A. montanum* and *H. radicata* plants showed significantly lower FRAP values (14% and 43% lower, respectively) than the respective Podzol-grown ones (Fig. 6). Among the Rendzina-grown plants, Fe-HBED treatment only significantly influenced FRAP values in *A. montanum* (16% increase; r25 vs r comparison) and *J. montana* (34% loss; r5 vs r comparison; Fig. 6). Almost all the tested parameters depended on the species, treatment and the interaction between these two factors; however, no significant effects were observed on *ortho*-dihydroxyphenolic or flavonoid content (Supplemental Table 9).

4. Discussion

Our findings indicated that dry acidic grasslands are composed of chlorosis-prone (*A. dioica*, *J. montana*) and chlorosis-resistant (*A. montanum*, *H. radicata*, *P. arenaria*) plant species. Additionally, the leaves of *H. radicata* and *P. arenaria* contained slightly less chlorophyll when the plants were grown on Rendzina than on Podzol, suggesting that the species that did not show evident chlorosis were also partially susceptible to Fe-dependent limitations. As the studied plants have different Fe demands, we rejected hypothesis 1. Thus, it appears that psammophilous calcifuges from European acidic grasslands do not constitute a homogenous ecological group in terms of Fe requirements (even species from the same family, e.g., *A. dioica* and *H. radicata*; question 1). A similar conclusion was drawn in the previous study on remarkable calcicoles from xerothermic alkaline grasslands (Wala et al., 2020).

Considering the growth of the studied plants, only *A. montanum* showed no evident preference for the studied substrata; all remaining species (*A. dioica*, *H. radicata*, *J. montana* and *P. arenaria*) showed a marked preference for the acidic soil. This result clearly confirms the calcifuge behavior of the investigated species, except for *A. montanum*. Similar growth inhibition on alkaline soil has been reported in the acidophilic *Rumex acetosella* L. (Zohlen and Tyler, 2000); however, unlike our present findings, some remarkable calcifuges, such as *Potentilla*

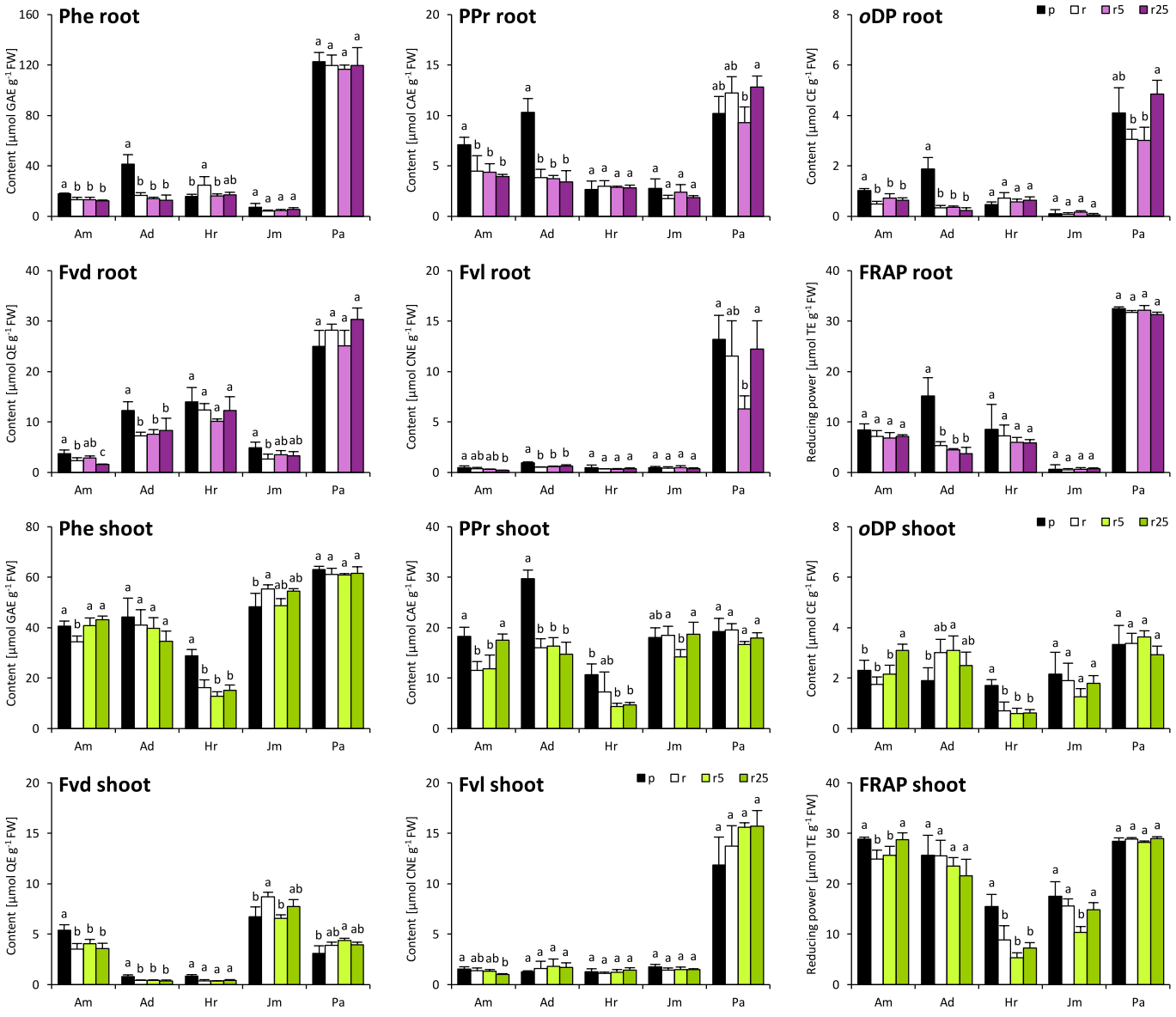

Fig. 6. Content of specialized metabolites (Phe – total phenolics, PPr – phenylpropanoids, *o*DP – *ortho*-dihydroxy phenolics, Fvd – total flavonoids and Fvl – flavanols) and antioxidative power (FRAP) in roots of the studied species grown on Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 µmol kg^{-1} soil Fe-HBED (r25). Values (mean ± SD) with the same letters for each parameter are not significantly different between the treatments within a given species at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD *post hoc* test).

argentea L. and the semiacidophilous *Viola riviniana* Rchb. and *Veronica officinalis* L., were not influenced by soil pH (Zohlen and Tyler, 2000). This suggests that European dry acidic grasslands are in fact composed of a mixture of plant specialists (stenotopic acidophiles) and generalists.

A. montanum, like some other plant species, such as *Centaurea stoebe* Tausch, appears to be a xerothermophilic generalist (Wala et al., 2021). It occurs on continental acidic species-rich grasslands (from *Koelerio glaucae-Corynephoretea* class) and grasslands resembling grass steppes (*Potentillo-Stipetum capillatae* Libb. 1933 em. Krausch 1960 association from *Festuco-Brometea* Br. Bl. et R. Tx. 1943 class; Matuszkiewicz, 2022). Although these plant communities grow on Ca-rich alkaline sands and soils originating from gypsum, our findings clearly indicated that *A. montanum* has a considerably wider niche. Additionally, *A. montanum* (as well as *P. arenaria*) readily colonizes dried-out post-mining soils known to be macronutrient-deficient and pH/heavy metal-limiting (Stefanowicz et al., 2016). This is in line with the conception that pH requirements can be of secondary importance for some plant species adapted to colonize dry sites (Palpurina et al., 2017).

The case of *P. arenaria* is also worth noting, as its reactions to the contrasting soils did not fully match the predictions based on previous data regarding its centre of abundance (especially R EIV). At first glance, *P. arenaria* inhabits a wide range of different habitats, including psammophilous grasslands (Czyżewska, 1992), steppe-like grasslands and even vegetation settled on karst (Kubíková, 1971; Kołodziejek et al., 2010; Albert et al., 2014); however, our findings indicate that its edaphic preference is definitely biased toward acidic soils. Such a preference for acidic soils is intuitively contradictory to its occurrence on bare Ca-rich rocky slopes; however, such slopes (e.g., in European Jura) and their vicinities (where stands of *P. arenaria* can be found) are often covered with superficial sandy acidic (calcite-free) allochthonous deposits, e.g., of glacial origin (Martignier et al., 2013). Thus, even bare shallow rocks can be in fact micromosaic habitats, where plants such as *P. arenaria* can find optimal environments for growth, i.e. allochthonous or semiallochthonous soils that are less alkaline than bedrock; such

examples can be found on the slopes of rocky outliers in Polish Jura (Kołodziejek et al., 2010). Interestingly, while both *P. arenaria* and *Salvia verticillata* L. are considered characteristic of grassland communities on dry and warm sites (from *Festuco-Brometea* class; Matuszkiewicz, 2022), the two species showed drastically different reactions to soil type (Wala et al., 2020). This suggests that such continental grasslands are highly mosaic with regard to both soil acidity and plant adaptation.

Hence, it appears that the soil preference of calcifuge plant species is a complex issue: their acidophilism can be strong or weak. It can also be overestimated or underestimated when only vegetation patterns are analyzed. However, our findings demonstrate that issues pertaining to the soil preferences of calcifuges can be successfully clarified using a controlled experimental setup.

The growth and development of all studied species grown on alkaline soil depended on Fe availability; however, the observed reaction patterns were species specific (question 1). The growth of *A. montanum*, *A. dioica* and *H. radicata* on alkaline soil was improved by treatment with chelated Fe (r25), indicating that Fe availability can be a limiting factor in their establishment. This is in agreement with the data concerning calcicole/calcifuge plant behavior (Gries and Runge, 1995; Zohlen and Tyler, 2000; Bothe, 2015). Nevertheless, although Fe application improved chlorophyll content (*P. arenaria*) and diminished chlorosis (*A. dioica* and *J. montana*) in the Rendzina-grown plants, it did not substituted fully acidic soil conditions. Although it is possible that the Fe doses were not enough to satisfy the Fe demands of the studied plants, some Fe-excess limitations associated with chlorophyll were observed (described below), which suggests the presence of other limiting factors.

Although our study does not conclusively determine what caused the growth impairments in alkaline soil, soil alkalinity, high Ca and carbonate availability or disturbances in the acquisition of selected micronutrients seem to be more probable explanations than P-dependent limitations (Tyler, 1992; Bothe, 2015; Strawn et al., 2020). From an environmental standpoint, it is rare to observe definite calcifuges on calcareous (or intentionally alkalized) soils, especially for individual plants with developed nutrient-dependent chlorosis. Such plants are most likely rapidly eliminated by other plant species or vanish due to the co-limitation of pH and/or Fe and other stressors. If so, alkalized or Fe-limiting sites within the dry acidic grassland micromosaic contribute to the patchy structure of vegetation. However, some plant–plant interactions may have positive effects by cross-solubilizing Fe, although this is very poorly understood in the case of acidic soils and dry acidic grasslands (Dai et al., 2019), and intraspecific adaptation to edaphic conditions such as alkalinity may also occur (Terés et al., 2019).

Our results indicated that chlorosis could be prevented by the application of chelated Fe (hypothesis 2); however, chlorophyll loss does not result solely from Fe starvation because plants grown on alkaline soil contained similar amounts of Fe, or even more, than plants grown on acidic soil. This also suggest that Fe-dependent chlorosis does not rely on the disrupted allocation of elements on the root-shoot axis, which is in agreement with our previous study (Wala et al., 2020). A recent point of view suggests that lime chlorosis is an impairment caused by complex interactions between certain mineral nutrients, such as Zn, Mn and P (Lešková et al., 2017; Therby-Vale et al., 2022) rather than by the simple inability of plants to acquire Fe (question 2). While the issue of lime chlorosis complexity is based on studies on model plants, our findings indicate that it is also valid for wild-living species (hypothesis 3 and question 3); indeed, increased acquisition of both Zn and Mn coincided with a detectable but chelate-reversible loss of chlorophyll content in *A. dioica* and *J. montana*. A similar reaction pattern was also observed in acidophilous *Ulmus laevis* Pall. (Venturas et al., 2014). Additionally, experimental oversupplementation of plants with both Mn and Zn was showed to be a factor responsible for induction of chlorosis closely mimicking Fe-dependent limitations (Lešková et al., 2017; Therby-Vale et al., 2022). This is also in agreement with previous findings indicating that Fe starvation in plants is associated with simultaneous, but probably pathophysiological, acquisition of Mn and Zn from the soil (White and Neugebauer, 2021). Unintended co-uptake of these elements during Fe scavenging depends on competition of elements for divalent ion transporters during acquisition (Briat et al., 2015a; Kaur and Garg, 2021; Therby-Vale et al., 2022). Thus, the fact that Fe:Mn and Fe:Zn ratios are much lower in Rendzina (c.a. 4.7 and 193.9, respectively) than in Podzol (>50.0 and >589.0; Wala et al., 2020) seems to be strongly involved in the development of chlorosis in the studied species, as chlorosis-susceptible plants are not able to avoid the toxicity of the Mn and Zn ions they acquire during Fe uptake. *In planta*, increased Zn uptake causes translocation of Mn from roots to shoots, and halts the translocation of Fe and contributes to the development of chlorosis (Kaur and Garg, 2021; Therby-Vale et al., 2022). This can be a serious problem for calcifuges, as they are naturally not adapted to elevated availability of Zn because the weathered acidic soils they inhabit contain lower amounts of Zn due to its greater removal than Fe, Mn and Al than in other soil types (Strawn et al., 2020). Excess Zn enters chloroplasts upon Fe starvation (Vigani et al., 2018) and is believed to disrupt chloroplast structure and impair chlorophyll maintenance; chlorophyll contents can be depressed due to degradation by chlorophyllase (CLH; EC 3.1.1.14) and lowered biosynthesis resulting from inhibition of δ-aminolevulinic acid dehydratase (ALAD; EC 4.2.1.24) and protochlorophyllide reductase (POR; EC 1.3.1.33) (Kaur and Garg, 2021). Similarly, excess of Mn reduces the abundance of numerous proteins, including those involved in the functioning of the photosynthetic apparatus, such as those associated with PSI and PSII (Liu et al., 2019); however, the full spectrum of molecular targets for Mn toxicity in chloroplasts remain unknown (Santos et al., 2017). All this suggests active involvement of Mn and Zn in development of Fe-dependent limitations (including lime chlorosis) rather than their irrelevance to formation of these impairments in plants. However, the degree of involvement of these two metals still needs further elucidation as they are also microelements pivotal for plant existence.

Some dicotyledonous plant species are able to cope with Zn and Mn toxicity by compartmenting excessive Mn in roots (Eroglu et al., 2016; Therby-Vale et al., 2022) or leaves (Küpper et al., 1999; Broadhurst et al., 2004), thus allowing efficient Fe acquisition and chlorophyll maintenance. While these mechanisms are probably partially involved in chlorosis resistance in *A. montanum*, *H. radicata* and *P. arenaria*, they are unlikely to be well developed in *A. dioica* and *J. montana*. Hence, the chlorosis observed in *A. dioica* and *J. montana* appears to result from non-optimal Fe:Mn and/or Fe:Zn ratios or even simple Mn toxicity: the content of Mn in the shoots of *A. dioica* was close to the threshold value that triggers Mn toxicity, i.e. around 150 μg g^{-1} DW, although in some Asterales, this value can be higher (Li et al., 2019; White and Neugebauer, 2021). Interestingly, chlorosis caused by Fe–Mn imbalance is believed to be associated with the disruption of Mg translocation (Li et al., 2019); however, our findings suggest that this mechanism was probably not involved in the development of chlorosis in the studied species.

The overall quantity of light energy converted into chemical energy per single plant is dependent on a range of internal species traits, environmental determinants and their interactions. The photosynthetic yield also depends partially on leaf number and structure (e.g., area, anatomy and functional status). Leaf weight, area and dimensions are influenced by various environmental factors, including the availability of Fe (Ding et al., 2019; Wala et al., 2020); however, the length and width of leaves can be affected proportionally or not (Shi et al., 2014; Wala et al., 2020). Fe availability affected leaf length more than leaf width in *J. montana* and *A. dioica*, which yielded stubby-leaved chlorotic plants. Most likely, this can be the result of multilayer reorganization in cells, including changes in the expression pattern of the cytochromes responsible for regulating leaf length (Tsukaya, 2005; Mai et al., 2016), or changes in Fe trafficking for heme and chlorophyll biosynthesis (Briat et al., 2015b). In contrast, while leaf width and length changed proportionally with soil Fe status in *A. montanum* and *H. radicata*, they

remained unaffected in *P. arenaria*. However, in all the tested species, a similar relationship between leaf area and edaphic conditions was observed, i.e., the studied calcifuges developed leaves with smaller area on non-optimal substratum (Rendzina) than under optimal edaphic conditions (Podzol). This is a significant observation, as smaller leaves scavenge less light and cover less area, and plants with such leafage can be eliminated by interference competition with neighboring well-adapted plants (Mortimer, 1992).

What is worse but totally expected, the leaves from chlorotic plants were not only smaller but also functionally defective. The *A. dioica* and *J. montana* grown on the alkaline substratum demonstrated elevated DI_0/RC and lowered F_V/F_M and PI_{ABS} as a result of Fe deficiency (chlorotic species). *P. arenaria* (nonchlorotic species) also showed a similar but less outlined reaction pattern when compared to *A. dioica*, *J. montana* or some chlorosis-prone calcicoles, e.g., *Aster amellus* L. or *Betonica officinalis* L. (Wala et al., 2020). A very similar reaction pattern has been observed in other plant species as a result of both simple Fe deficiency (Prity et al., 2020) and more complex limitations caused by soil alkalinity (Luna et al., 2018). Interestingly, some Fe-excess-dependent limitations were also observed in the present study. For example, negative changes in chlorophyll *a* fluorescence were observed in *H. radicata*, which coincided with gradual chlorophyll loss, following the application of Fe-HBED. This was contrary to our expectations but in agreement with other investigations reporting detrimental changes in chlorophyll *a* fluorescence associated with excess Fe (e.g., for F_V/F_M, ET_0/RC, DI_0/RC, PI_{ABS}) in *Ipomoea batatas* (L.) Lam. (Adamski et al., 2011). It was surprising to observe such negative changes in chlorophyll *a* fluorescence in this species, considering the observed growth relations. This contradiction most likely resulted from the Fe application causing an initial nutritional boost for growth, which was later followed by a gradual worsening of plant homeostasis due to Fe intoxication and oxidative stress (Adamski et al., 2011). *J. montana* showed better performance on Podzol than on Rendzina; however, it showed poorer photosynthetic function on Rendzina following Fe-HBED application, although correction of chlorosis was observed. It is possible that this could be due to the observed Cu shortage in the shoots (e.g., in chloroplasts): elevated Fe acquisition is known to act antagonistically with Cu acquisition in some species (e.g., in *Ocimum basilicum* L.; Adiloğlu, 2021) and the Fe-HBED-supplied *J. montana* plants contained less Cu in shoots than plants grown on untreated Rendzina or Podzol. It is possible, then, that Cu deficiency affected the pool of plastocyanin (Cu-containing protein), whose abundance is known to be reduced by lowered Cu acquisition (while F_V/F_M is not affected; Shahbaz et al., 2015). In summary, mineral nutrient signaling appears to control not only chlorophyll biosynthesis, but also other traits involved in photochemical capacity, such as leaf area (Therby-Vale et al., 2022). However, the influence of nutritional imbalances on plant functioning is a species-specific issue. Therefore, it can be proposed that the micromosaic structure of dry acidic grasslands (in terms of pH and nutrient availability) influences the spatiotemporal process of plant assembly by exerting a species-selective pressure on, widely-defined, photosynthetic capacity.

The studied calcifuges did not show any signs of Fe deficiency on acidic soil; this can be attributed to the acidity of Podzol, which increases Fe availability (Strawn et al., 2020) or the malic acid-based mechanism of P and Fe dissolution (Balzergue et al., 2017; Godon et al., 2019). When plants grow on alkaline soil, they employ alternative mechanisms based around the exudation of phenolic compounds into the rhizosphere (Zwetsloot et al., 2018; Dan et al., 2020). These specialized metabolites obtained from the exudation and decomposition of plant debris improve the availability of trace elements, including Fe, due to selective or nonselective chelation, depending on their chemical structure (Wasli et al., 2018). Although exudation was not analyzed in this study, the amount of specialized metabolites in roots are known to correlate with their exudation (Tato et al., 2013; Zwetsloot et al., 2018). Thus, it can be expected that species with low biosynthesis of specialized metabolites when grown on alkaline soil (*J. montana* and *A. dioica*) release lower amounts. Additionally, *A. dioica* showed a collapse of specialized metabolite biosynthesis when grown on alkaline substratum, which probably contributed to its chlorosis susceptibility; this is contrary to the observations on *Anethum graveolens* L. (Wasli et al., 2018). The FRAP method, an effective indicator of antioxidant power, can also provide valuable information about the approximate ability of the analyzed constituents to reduce Fe. As phenolic compounds synthesized by plants can both chelate and reduce Fe (Nkhili et al., 2014 and articles cited therein) and FRAP values correspond well with the contents of specialized metabolites, it can be proposed that *A. dioica* and *J. montana* develop pH-dependent chlorosis in response to Fe shortages; this may be due to their poor ability to produce and/or exude specialized metabolites needed for Fe chelation and/or reduction under alkaline conditions. In contrast, it is possible that the chlorosis-resistant species (*A. montanum*, *H. radicata* and *P. arenaria*) are able to satisfy their Fe demands on contrasting soils by secreting malate-based (on acidic soil) and phenolic-based (on alkaline soil) substances to dissolve Fe or switch between them efficiently. For example, *P. arenaria* showed constant, strong biosynthesis of phenolic compounds when compared to other species, especially flavanols, whose biosynthesis is known to be enhanced in the *Rosaceae* (Hoffmann et al., 2012). Although the contents of the studied metabolites determined in *A. montanum* and *H. radicata* were definitely lower than those in *P. arenaria*, these species were also able to satisfy their nutritional demands. It must be mentioned here that the structure, and thus charge density at the chelation site of each metabolite, shapes its chelation capacity (Andjelković et al., 2006). Additionally, specialized phenolic metabolites synthesized by plants represent high structural diversity and are subjected to different fates in soil environment. Therefore, generalization in this subject is very difficult because data are still very scarce and originate from laboratory investigations (Cesco et al., 2012; Tsai and Schmidt, 2017). Hence, there is a need for further studies on the role of specialized metabolism, and its qualitative and quantitative changes based on soil alkalinity (Robe et al., 2021).

Interestingly, from the biochemical point of view, soil type and Fe seemed to influence the different metabolites within a given species to similar degrees, with a slight exception for *P. arenaria*; this suggests that the specialized metabolism may be inhibited at the committed step of the phenylpropanoid pathway, e.g., on phenylalanine ammonia lyase (PAL; EC 4.3.1.24), due to specific regulation of its activity or substrate shortage (Wasli et al., 2018).

The biosynthesis of phenolic compounds is believed to be a generalized stress response. In addition to their structural role, many phenolics act as antioxidants, and they are commonly used to scavenge free radicals (Naikoo et al., 2019). Many plant species exposed to Fe starvation or soil alkalinity show enhanced biosynthesis of phenolics in leaves (Wasli et al., 2018; Naikoo et al., 2019); however, this was not the case in the studied calcifuges (except *J. montana*), as *A. montanum*, *A. dioica* and *H. radicata* showed species-specific downregulation of specialized metabolite biosynthesis when kept on the non-optimal substratum. In contrast, the specialized metabolism in *P. arenaria* seemed to be independent of edaphic conditions, which highlights the diversity of effects of soil properties on specialized metabolism in calcifuges. Under alkalinity stress, the studied species failed to initiate any systemic response involving increased biosynthesis of specialized metabolites, and demonstrated nutritional (elemental) limitations; this strongly suggests worsened functioning, which can make them susceptible to both abiotic and biotic stressors. This may be of great importance for xerothermophilic plant species because they must rely on effective photoprotection and maintenance of structural integrity.

In conclusion, the present investigation showed that the dry acidic grasslands are composed of chlorosis-susceptible (e.g., *A. dioica* and *J. montana*) and chlorosis-resistant species (e.g., *A. montanum*, *H. radicata* or *P. arenaria*). This indicates that their similar requirements for substratum acidity are counterbalanced by their different

requirements for Fe availability. Therefore, Fe-based niche differentiation implies that in this group, resistance to Fe limitations should not be estimated solely based on pH requirements, and *vice versa*. Moreover, pH-dependent limitations in calcifuges are associated not only with disrupted Fe acquisition, but are also influenced by the availability of other microelements (Mn and Zn). Sufficient Fe supplementation on the alkaline substratum with Fe-HBED did not fully replicate the conditions that the studied species naturally encounter on acidic soil, which suggests other sources of limitations. Hence, Fe deficiency has a complex etiology, and to gain an insight into the role of the environment in shaping the Fe starvation problem at the level of multispecies assemblages, further research outside laboratory studies on non-model plants is required.

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CRediT authorship contribution statement

Mateusz Wala: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Jeremi Kołodziejek:** Resources, Writing – review & editing, Supervision. **Janusz Mazur:** Methodology, Investigation.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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P–2

Materiał uzupełniający

(Supplementary material)

Supplemental Table 1. Values indicating optimal environmental conditions for vegetative growth the studied species.

Indicator values follows 1–9 scale proposed by Ellenberg et al. (1991). L: light requirements ranging 7–9, where 7 indicates well-lit/slightly-shaded conditions (c.a. 30% of relative illumination) and 9 indicates full light conditions (> 50% of relative illumination); T: temperature requirements with value ranging 5–7, where 5 indicates moderately cool to warm conditions (characteristic for montane and submontane locations, mostly Fennoscandia) and 7 indicates warm conditions (characteristic for North European Plain); K: continentality requirements ranging 3–6, where 3 indicates atlantic/subatlantic conditions and 6 indicates subatlantic to subcontinental conditions; F: soil moisture requirements ranging 1–5, where 1 indicates extremely dry soils and 5 indicates moist soils; R: soil pH requirements ranging 3–8, where 3 indicates acidic soils and 8 indicate basic soils; N: soil nitrogen requirements ranging 1–3, where 1 indicates extremely infertile soils and 3 indicates slightly fertile soils. 0 – wide amplitude or unequal behaviour in different areas with the same conditions.

Species	Ellenberg's indicator value					
	Light (L)	Temperature (T)	Continent (K)	Moisture (F)	pH (R)	Nitrogen (N)
Am	9	6	4	2	7	1
Ad	8	0	0	4	3	2
Hr	8	5	3	5	4	3
Jm	7	6	3	3	3	2
Pa	9	7	6	1	8	1

Supplemental Table 2. Parameters derived from the OJIP transient used in this study, formulas of their calculation and definitions. Data presented in table follows Wala et al. (2020).

OJIP parameter	Formula	Definition
F_0 (=F_O)	F_0=F at 50μs (=F at O-step)	Fluorescence intensity at O-step (at 50 μs) (=Minimal fluorescence intensity)
F_J	F_J=F at 2ms (=F at J-step)	Fluorescence intensity at J-step (at 2 ms)
F_I	F_I=F at 60ms (=F at I-step)	Fluorescence intensity at I-step (at 60 ms)
F_M (=F_P)	F_M=F at 1s (=F at P-step)	Fluorescence intensity at P-step (at 1000 μs) (=Maximal fluorescence intensity)
F_V	$F_V=F_M–F_0$	Maximal variable fluorescence
V_J	$V_J=(F_J–F_0)/(F_M–F_0)$	Relative variable fluorescence at J-step (2 ms)
V_I	$V_I=(F_I–F_0)/(F_M–F_0)$	Relative variable fluorescence at I-step (60 ms)
F_V/F_M	-	Maximum quantum yield of primary PSII photochemistry
M_0	$M_0=TR_0/RC–ET_0/RC$	Approximated initial slope of the fluorescent transient
Area	-	Area between fluorescence curve and F_M (background subtracted)
S_M	S_M=Area/(F_M - F_0)	Standardized area above the fluorescence curve between F_0 and F_M
N	$N=S_M*M_0*(1/V_J)$	Number of Q_A redox turnovers until F_M is reached
φ_{E0}	$\varphi_{E0}=[1–(F_0/F_M)]*\psi_0$	Quantum yield for electron transport from Q_A to plastoquinone at t = 0
PI_{ABS}	$PI_{ABS}=\gamma RC/(1-\gamma RC)*\varphi_{P0}/(1-\varphi_{P0})*\psi_0/(1-\psi_0)$	Performance index of electron flux from PSII based to intersystem acceptors
ABS/RC	$ABS/RC=M_0*(1/V_J)*(1/\varphi_{P0})$	Photon flux absorbed by PSII antenna chlorophyll per RC at t = 0
TR_0/RC	$TR_0/RC=M_0*(1/V_J)$	Trapping flux leading to Q_A reduction per RC at t = 0
ET_0/RC	$ET_0/RC=M_0*(1/V_J)*\psi_0$	Electron transport flux per RC at t = 0
DI_0/RC	$DI_0/RC=(ABS/RC)–(TR_0/RC)$	Dissipated energy flux per RC at t = 0

Supplemental Table 3. Results of two-way ANOVA (*F* values and significance) showing effects of species, treatment and their interaction on the chlorosis related parameters. Differences considered as statistically significant ($p < 0.05$) were bolded.

Parameter	*F* values and significance		
	(S) Species (*df* = 4)	(T) Treatment (*df* = 3)	S x T (*df* = 12)
Chlorophyll content	**168.1 (<0.001)**	**75.1 (<0.001)**	**19.9 (<0.001)**
IDC score	**20.3 (<0.001)**	**31.4 (<0.001)**	**14.8 (<0.001)**

Supplemental Table 4. Results of two-way ANOVA (*F* values and significance) showing effects of species, treatment and their interaction on the growth related parameters. Differences considered as statistically significant ($p < 0.05$) were bolded.

Factor	*F* values and significance		
	(S) Species (*df* = 4)	(T) Treatment (*df* = 3)	S x T (*df* = 12)
Root FW	**101.6 (<0.001)**	**9.9 (<0.001)**	**5.3 (<0.001)**
Root DW	**97.5 (<0.001)**	**8.4 (<0.001)**	**4.1 (<0.001)**
Shoot FW	**218.4 (<0.001)**	**32.7 (<0.001)**	**11.7 (<0.001)**
Shoot DW	**72.1 (<0.001)**	**25.6 (<0.001)**	**5.5 (<0.001)**
FW shoot:root ratio	**13.6 (<0.001)**	**0.9 (0.028)**	**1.6 (<0.001)**
DW shoot:root ratio	**37.9 (<0.001)**	**3.3 (0.026)**	**4.4 (<0.001)**
Leaf length	**407.3 (<0.001)**	**31.9 (<0.001)**	**8.4 (<0.001)**
Leaf width	**411.5 (<0.001)**	**6.4 (<0.001)**	**2.9 (<0.001)**
Leaf area	**392.0 (<0.001)**	**12.7 (<0.001)**	**6.3 (<0.001)**
Leaf perimeter	**257.0 (<0.001)**	**18.3 (<0.001)**	**6.6 (<0.001)**
Leaf length:width ratio	**192.4 (<0.001)**	**6.7 (<0.001)**	**5.2 (<0.001)**

Supplemental Table 5. Leaf morphometric parameters of the studied species grown in Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 µmol kg^{-1} soil Fe-HBED (r25). Values (mean ± SD) with different letters for each parameter are significantly different between the treatments at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD post-hoc test).

Species	Treatment	Lenght [cm]	Width [cm]	Area [cm^2]	Perimeter [cm]	Length:Width Ratio
Am	p	4.79 ± 0.60b	1.12 ± 0.11b	2.81 ± 0.56b	14.80 ± 1.64b	4.30 ± 0.15a
	r	4.35 ± 0.36b	1.01 ± 0.09b	2.26 ± 0.36b	13.23 ± 1.45b	4.35 ± 0.39a
	r5	5.17 ± 0.21b	1.18 ± 0.07b	3.02 ± 0.15b	15.09 ± 0.83b	4.42 ± 0.33a
	r25	6.63 ± 0.57a	1.47 ± 0.06a	4.89 ± 0.76a	19.58 ± 1.51a	4.50 ± 0.22a
Ad	p	2.47 ± 0.14a	1.07 ± 0.04b	1.34 ± 0.10b	6.30 ± 0.31a	2.31 ± 0.05a
	r	1.93 ± 0.16b	0.99 ± 0.03c	1.12 ± 0.08c	5.19 ± 0.35b	1.94 ± 0.13c
	r5	2.25 ± 0.07a	1.15 ± 0.04a	1.43 ± 0.06ab	6.06 ± 0.23a	1.96 ± 0.04bc
	r25	2.52 + 0.19a	1.17 ± 0.03a	1.61 ± 0.14a	6.60 ± 0.49a	2.16 ± 0.15ab
Hr	p	10.34 ± 1.19a	3.34 ± 0.55a	16.85 ± 3.29a	42.49 ± 6.13a	3.26 ± 0.82a
	r	7.23 ± 1.07b	2.43 ± 0.25b	9.97 ± 1.97b	24.66 ± 3.68b	3.00 ± 0.37a
	r5	8.61 ± 0.25ab	2.76 ± 0.21ab	13.13 ± 1.04ab	30.40 ± 3.24b	3.15 ± 0.26a
	r25	8.72 ± 0.59ab	3.09 ± 0.32ab	15.75 ± 2.09a	31.21 ± 5.25b	2.84 ± 0.29a
Jm	p	5.73 ± 0.22a	0.69 ± 0.16a	1.54 ± 0.27a	14.09 ± 0.83a	8.71 ± 1.54a
	r	2.82 ± 0.27c	0.60 ± 0.08a	0.83 ± 0.08b	7.43 ± 1.03c	4.78 ± 0.38b
	r5	4.16 ± 0.07b	0.63 ± 0.05a	1.08 ± 0.14b	11.11 ± 1.55b	6.75 ± 0.40ab
	r25	4.59 ± 0.73b	0.58 ± 0.11a	1.11 ± 0.23b	11.66 ± 1.64ab	8.27 ± 2.33a
Pa	p	3.05 ± 0.54a	3.24 ± 0.48a	3.77 ± 1.03a	23.32 ± 3.51a	0.94 ± 0.04a
	r	2.89 ± 0.08a	3.06 ± 0.14a	3.40 ± 0.35a	21.48 ± 1.53a	0.94 ± 0.03a
	r5	2.72 ± 0.33a	2.98 ± 0.36a	3.48 ± 0.29a	21.76 ± 1.28a	0.92 ± 0.05a
	r25	2.84 ± 0.27a	3.00 ± 0.28a	3.35 ± 0.55a	21.62 ± 2.80a	0.95 ± 0.01a

Supplemental Table 6. Results of two-way ANOVA (*F* values and significance) showing effects of species, treatment and their interaction on the OJIP-related parameters. Differences considered as statistically significant ($p < 0.05$) were bolded.

Parameter	*F* values and significance		
	(S) Species (*df* = 4)	(T) Treatment (*df* = 3)	S x T (*df* = 12)
F_0	**12.5 (<0.001)**	**4.3 (0.006)**	**3.3 (<0.001)**
F_J	**32.2 (<0.001)**	**3.3 (0.024)**	**3.8 (<0.001)**
F_I	**32.4 (<0.001)**	2.1 (0.103)	**3.7 (<0.001)**
F_M	**45.6 (<0.001)**	**3.5 (0.018)**	**3.8 (<0.001)**
F_V	**54.8 (<0.001)**	**3.7 (0.014)**	**4.2 (<0.001)**
V_J	**70.3 (<0.001)**	**10.9 (<0.001)**	**7.0 (<0.001)**
V_I	**30.2 (<0.001)**	**3.1 (0.030)**	**4.9 (<0.001)**
F_V/F_M	**40.4 (<0.001)**	**7.2 (<0.001)**	**4.6 (<0.001)**
M_0	**25.8 (<0.001)**	**8.4 (<0.001)**	**4.7 (<0.001)**
Area	**5.1 (<0.001)**	**3.1 (0.029)**	**2.3 (0.012)**
S_M	**13.5 (<0.001)**	**4.8 (0.003)**	**1.9 (0.040)**
N	**11.8 (<0.001)**	**3.3 (0.023)**	1.4 (0.193)
φ_{E0}	**55.6 (<0.001)**	**9.5 (<0.001)**	**4.3 (<0.001)**
PI_{ABS}	**22.8 (<0.001)**	**12.3 (<0.001)**	**6.1 (<0.001)**
ABS/RC	**32.3 (<0.001)**	**13.1 (<0.001)**	**5.0 (<0.001)**
TR_0/RC	**34.2 (<0.001)**	**14.5 (<0.001)**	**5.0 (<0.001)**
ET_0/RC	**101.8 (<0.001)**	**5.8 (<0.001)**	**7.9 (<0.001)**
DI_0/RC	**49.0 (<0.001)**	**21.9 (<0.001)**	**10.9 (<0.001)**

Supplemental Table 7. Results of two-way ANOVA (*F* values and significance) showing effects of species, treatment and their interaction on the elemental composition parameters. Differences considered as statistically significant ($p < 0.05$) were bolded.

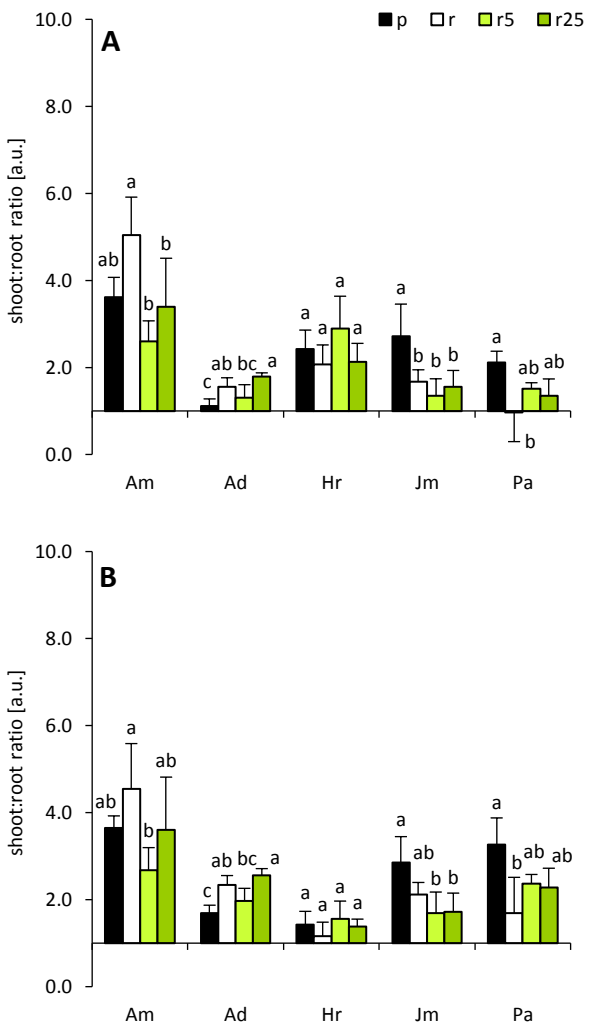
Trait	Factor	*df*	*F* values and significance					
			Ca	Mg	Fe	Mn	Zn	Cu
Root content	(S) Species	4	**9.2 (<0.001)**	**136.0 (<0.001)**	**6.1 (<0.001)**	**24.2 (<0.001)**	**167.4 (<0.001)**	**16.5 (<0.001)**
	(T) Treatment	3	**7.0 (<0.001)**	1.8 (0.161)	**8.0 (<0.001)**	**20.3 (<0.001)**	**10.0 (<0.001)**	1.0 (0.391)
	S x T	12	**3.2 (0.001)**	**6.4 (<0.001)**	**4.7 (<0.001)**	**7.5 (<0.001)**	**3.9 (<0.001)**	**7.6 (<0.001)**
Shoot content	S	4	**89.3 (<0.001)**	**56.6 (<0.001)**	**10.1 (<0.001)**	**87.7 (<0.001)**	**51.3 (<0.001)**	**20.3 (<0.001)**
	T	3	1.7 (0.169)	**45.9 (<0.001)**	**7.9 (<0.001)**	**52.0 (<0.001)**	**8.9 (<0.001)**	**3.9 (0.013)**
	S x T	12	**6.8 (<0.001)**	**8.4 (<0.001)**	0.8 (0.632)	**28.9 (<0.001)**	**2.1 (0.028)**	**7.0 (<0.001)**
SAP	S	4	**16.5 (<0.001)**	**49.1 (<0.001)**	**6.5 (<0.001)**	**12.8 (<0.001)**	**133.9 (<0.001)**	**9.3 (<0.001)**
	T	3	**9.5 (<0.001)**	**10.6 (<0.001)**	**7.3 (<0.001)**	**16.7 (<0.001)**	2.1 (0.116)	**4.9 (0.004)**
	S x T	12	**4.2 (<0.001)**	**7.7 (<0.001)**	**4.0 (<0.001)**	**7.0 (<0.001)**	**4.2 (<0.001)**	**7.4 (<0.001)**

Supplemental Table 8. Results of two-way ANOVA (*F* values and significance) showing effects of species, treatment and their interaction on the Fe:Mn and Fe:Zn ratios in roots and shoots. Differences considered as statistically significant ($p < 0.05$) were bolded.

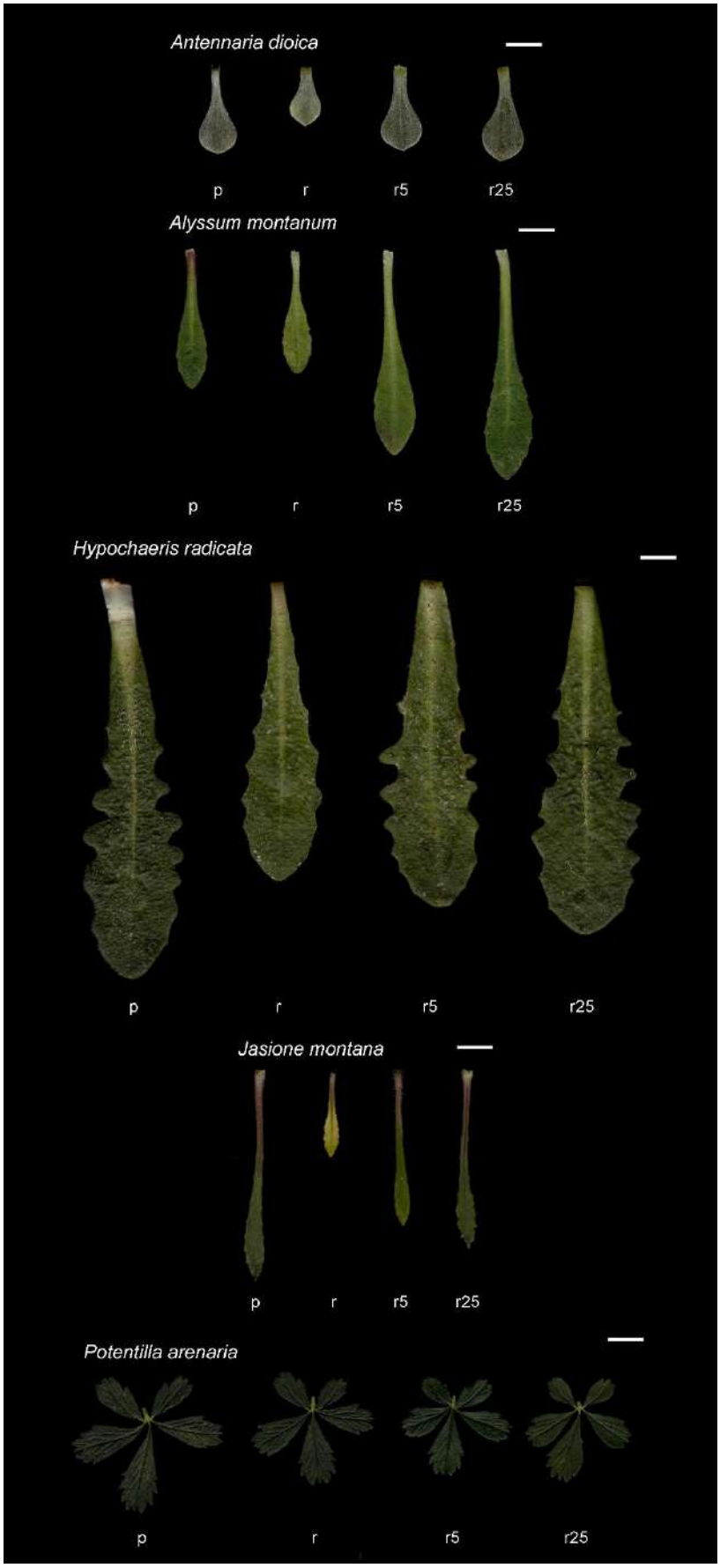
Parameter	*F* values and significance		
	(S) Species (*df* = 4)	(T) Treatment (*df* = 3)	S x T (*df* = 12)
Fe:Mn Roots	**10.4 (<0.001)**	**4.0 (0.011)**	**10.1 (<0.001)**
Fe:Mn Shoots	**23.2 (<0.001)**	**12.8 (<0.001)**	**5.9 (<0.001)**
Fe:Zn Roots	**37.2 (<0.001)**	**10.3 (<0.001)**	**5.6 (<0.001)**
Fe:Zn Shoots	**15.3 (<0.001)**	2.5 (0.067)	0.9 (0.521)

Supplemental Table 9. Results of two-way ANOVA (*F* values) showing effects of species, treatment and their interaction on the content of specialized metabolites and antioxidative power.
Differences considered as statistically significant ($p < 0.05$) were bolded.

Parameter	*F* values and significance		
	(S) Species (*df* = 4)	(T) Treatment (*df* = 3)	S x T (*df* = 12)
Root phenolics	**1462.5 (<0.001)**	**10.8 (<0.001)**	**6.0 (<0.001)**
Root phenylpropanoids	**208.3 (<0.001)**	**17.0 (<0.001)**	**12.5 (<0.001)**
Root *ortho*-dihydroxyphenolics	**319.5 (<0.001)**	**14.3 (<0.001)**	**10.2 (<0.001)**
Root flavonoids	**536.5 (<0.001)**	**5.8 (0.002)**	**4.2 (<0.001)**
Root flavanols	**249.2 (<0.001)**	**6.1 (0.001)**	**5.4 (<0.001)**
Root FRAP	**883.3 (<0.001)**	**17.1 (<0.001)**	**7.4 (<0.001)**
Shoot phenolics	**378.1 (<0.001)**	**6.4 (<0.001)**	**6.9 (<0.001)**
Shoot phenylpropanoids	**108.6 (<0.001)**	**40.7 (<0.001)**	**10.4 (<0.001)**
Shoot *ortho*-dihydroxyphenolics	**63.2 (<0.001)**	0.4 (0.786)	**5.4 (<0.001)**
Shoot flavonoids	**726.4 (<0.001)**	1.4 (0.249)	**11.0 (<0.001)**
Shoot flavanols	**643.5 (<0.001)**	**3.1 (0.033)**	**3.5 (0.001)**
Shoot FRAP	**291.4 (<0.001)**	**18.4 (<0.001)**	**4.9 (<0.001)**



Supplemental Figure 1. Fresh weight (FW; A) and dry weight (DW; B) partitioning measured as shoot:root ratio of the studied calcifuge species grown in Podzol (p), Rendzina (w) or Rendzina with addition of 5 (r5) or 25 µmol kg^{-1} soil Fe-HBED (r25). Values (mean ± SD) with different letters for each parameter are significantly different between the treatments at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD post-hoc test).



Supplemental Figure 2. Appearance of leaves of the studied calcifuge species grown in Podzol (p), Rendzina (w) or Rendzina with addition of 5 (r5) or 25 µmol kg^{-1} soil Fe-HBED (r25). The images shows representative leaves sampled for evaluation of leaf morphometrical traits. Each bar is equal to 1 cm.

Praca P–3

W trzecim etapie prac skupiono się na gatunkach współwystępujących w obu typach siedlisk. Do podjęcia tematu przyczyniły się przede wszystkim wyniki z poprzednich etapów badań, wskazujące na zróżnicowanie preferencji substratowej oraz wymagań względem dostępności żelaza u roślin występujących w obu typach muraw. Pomimo obserwowanego zróżnicowania reakcji roślin należących do tej samej rodziny (Lamiaceae w obrębie gatunków z kserotermicznych muraw wapieniolubnych i Asteraceae z piaszczystych muraw bezwapiennych), nie było jasne, czy rośliny bardzo blisko spokrewnione (z tego samego rodzaju) i występujące w obrębie obu badanych typów muraw wykazują takie same (lub bardzo zbliżone) reakcje względem zmieniających się warunków edaficznych (wysoki stopień wzajemnego pokrycia nisz), czy też ich reakcje te są różne (niskie pokrycie wzajemne nisz). Do badań wybrano *C. scabiosa* i *C. stoebe* tworzące w założeniu kongeneryczną parę gatunków. Wybór podyktowany był opisami przedstawianymi w pracach z zakresu fitosocjologii, w których to oba gatunki były wymieniane jako współwystępujące w obrębie różnych typów muraw oraz wynikami wcześniejszych prac własnych wskazujących na brak międzygatunkowych różnic w wybranych wymiarach ich nisz ekologicznych (np. jakościowe i ilościowe wymagania względem dostępności azotu w kontekście kiełkowania; Wala i wsp., 2021).

Do badań zastosowano tożsamy układ doświadczalny jak dla dwóch poprzednich prac oraz, podobnie jak w pracy P–2, odtworzono wszystkie procedury doświadczalne zastosowane w pracy P–1.

Zakres części testowanych hipotez (hipoteza H2, H3 i H4) był spójny z poprzednimi etapami badań. Modyfikacji uległa pierwsza hipoteza badawcza, której nadano brzmienie: badane gatunki nie mają wyraźnych preferencji względem gleby (rosną równie dobrze na kwaśnej glebie bielicowej, jak i na zasadowej rędzinie właściwej; porównanie wariantów „p” i „r”; H1). Ponadto, sformułowano hipotezę piątą: wymaganie względem dostępności żelaza (reakcja na dostępność żelaza w glebie zasadowej) jest czynnikiem różnicującym oba gatunki (międzygatunkowy stosunek wartości jednego lub wielu parametrów wzrostu będzie wykazywał tendencję wzrostową lub spadkową w sekwencji „r”, „r5” i „r25”, a przynajmniej część różnic będzie istotna; H5), oraz hipotezę szóstą: pobór lub wzorzec alokacji jednego lub kilku pierwiastków różnicuje nisze ekologiczne badanych gatunków, niezależnie od gleby, na której rosną (międzygatunkowy stosunek wartości parametrów opisujących zawartość lub alokację pierwiastka jest istnie różny od jedności; porównanie wariantów „p”, „r”, „r5” i „r25”; H6).

Wyniki wskazały na brak istotnych różnic we wzroście badanych gatunków uprawianych na kontrastujących typach gleb. Oznacza to, że nie ma jasnych przesłanek do odrzucenia pierwszej hipotezy badawczej (H1). Można zatem przyjąć, że *C. scabiosa* i *C. stoebe* posiadają szeroki zakres tolerancji względem odczynu gleb suchych. Są to zatem rośliny o podwójnie generalistycznej strategii życiowej – zarówno w odniesieniu kserotermiczności siedlisk, jak i ich alkalizacji. Ze względu na powyższe, gatunki te mogą być uznane za rośliny rzeczywiście kongeneryczne.

Zarówno *C. scabiosa*, jak i *C. stoebe* nie wykazywały jakichkolwiek objawów niedoborów mineralnych, w tym przede wszystkim nie zaobserwowano fenotypowych objawów niedoborów żelaza. Niezależnie od wariantu, zawartość chlorofilu w liściach nie uległa zmianie, a zmiany parametrów związanych z fluorescencją chlorofilu *a* miały łagodny charakter w porównaniu do gatunków badanych w poprzednich etapach (np. *A. amellus*, *B. officinalis*, *P. grandiflora*, *A. dioica* i *J. montana*). Było to zgodne z ilościową analizą zawartości pierwiastków, w której nie notowano problemów z poborem żelaza na glebie zasadowej. Wyniki nie dały zatem podstaw do odrzucenia drugiej hipotezy badawczej (H2) oraz pozwoliły na odrzucenie hipotezy trzeciej i czwartej (H3 i H4); należy przez to przyjąć, że oba gatunki nie doznają ograniczeń związanych z gospodarką mineralną gdy wzrastają na podłożu zasadowym i nie cierpią z powodu zaburzeń związanych z niedoborem żelaza.

Doświadczalne nawożenie roślin żelazem wywołało efekty specyficzne gatunkowo – podczas gdy nie odnotowano istotnych różnic w podstawowych parametrach wzrostowych u *C. scabiosa* (choć trend zmian wskazuje że gatunek ten prawdopodobnie nie toleruje stężeń wyższych niż badane), u *C. stoebe* obserwowano istotną, zależną od zastosowanej dawki poprawę wzrostu. Miało to przełożenie na spadek międzygatunkowego stosunku wartości (*C. scabiosa* do *C. stoebe*) wybranych parametrów, tj. świeżej i suchej masy korzeni i części nadziemnych roślin oraz świeżej i suchej masy, liczby i powierzchni liści. Jednocześnie odnotowano, że dwa parametry opisujące liście: LDMC (ang. *leaf dry matter content*) oraz SLA (ang. *specific leaf area*), uznawane za tzw. parametry funkcjonalne o szerokim zastosowaniu w pracach z zakresu ekologii (Pierce i wsp., 2017), nie tłumaczą różnic między badanymi gatunkami. Jest to spójne z wynikami prac pokazującymi brak koordynacji między (na pozór) jeszcze bardziej związanymi ze sobą parametrami jak SLA i LDMC i punkt utraty turgoru (ang. *turgor loss point*, π_{TLP}; parametr opisujący zdolność roślin do utrzymania prawidłowego ciśnienia osmotycznego komórek w trakcie postępującego odwodnienia; Májeková i wsp., 2021). Dodatkowo, biorąc pod uwagę wyniki dwuczynnikowego testu ANOVA obejmującego wszystkie badane warianty, świeża masa części nadziemnych roślin oraz świeża i sucha masa liści wydają się najlepiej tłumaczyć różnice w preferencjach substratowych obu gatunków, wskazując, że *C. stoebe* wymaga większej dostępności żelaza w glebie niż *C. scabiosa*. Wyniki nie dały zatem powodu do odrzucenia hipotezy piątej (H5); należy przez to przyjąć, że zapotrzebowanie na żelazo odgrywa istotną rolę w różnicowaniu nisz obu gatunków.

Dane wskazują, że *C. scabiosa* pobiera istotnie więcej wapnia i cynku niż *C. stoebe* i w większym stopniu alokuje oba te pierwiastki do części nadziemnej, co uniemożliwia odrzucenie hipotezy szóstej (H6). Sugeruje to istnienie odmiennych wzorców zapotrzebowania na wapń i cynk u badanych gatunków. Ponadto, różnice międzygatunkowe notowano niezależnie od typu gleby, na której rosły rośliny. Co więcej, niższe zapotrzebowanie na niektóre pierwiastki (wapń i cynk), wyższa tolerancja na rosnącą dostępność żelaza w glebie oraz wyższe wartości SLA mogą przynajmniej częściowo tłumaczyć inwazyjność *C. stoebe*. Dane odnośnie preferencji badanych gatunków okazały się być zgodne z ostatnimi badaniami nad stepami serpentynitowymi zlokalizowanymi w obrębie Masywu Czesko-Morawskiego (Mrázková-Štýbnarová i wsp., 2021).

W kontekście poznawczym, niniejsze badanie wskazuje na istnienie różnic w niszach ekologicznych badanych gatunków (również w ujęciu gatunek nieinwazyjny – *C. scabiosa* i gatunek inwazyjny – *C. stoebe*) oraz, szerzej, wskazuje te aspekty w fizjologii, które mogą różnicować gatunki kongeneryczne. Z praktycznego punktu widzenia, powyższe informacje mogą posłużyć do wypracowywania skuteczniejszych strategii planowania i ewaluacji działań zagospodarowania i rekultywacji terenów, których stan gleb (oligotrofizm, kserotermizm, alkalizacja i/lub toksyczność spowodowana zwiększoną dostępnością pierwiastków) jest problematyczny, gdyż oba gatunki znane są z występowania na trudnych pod względem warunków edaficznych substratach (Woch i wsp., 2016; Mrázková-Štýbnarová i wsp., 2021).

W niniejszej części pracy doktorskiej zastosowano jedną metodę doświadczalną (częściowo kontrolowane doświadczenie polowe) oraz cztery techniki analityczne (metody wagowe, laserowy skaning optyczny, spektrofluorymetria oraz spektroskopia absorpcji atomowej). W oparciu o uzyskane wyniki opublikowano autorską pracę w czasopiśmie *PeerJ* zatytułowaną „*Reactions of two xeric-congeneric species of Centaurea (Asteraceae) to soils with different pH values and iron availability*”.

P–3

"Reactions of two xeric-congeneric species of Centaurea *(Asteraceae) to soils with different pH values and iron availability"*

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Reactions of two xeric-congeneric species of *Centaurea* (Asteraceae) to soils with different pH values and iron availability

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ABSTRACT

Centaurea scabiosa L. and *C. stoebe* Tausch are known to co-exist naturally in two extremely different types of open dry habitats in the temperate zone, alkaline xerothermic grasslands and acidic dry grasslands. However, knowledge about their preferences to edaphic conditions, including soil acidity (pH), and iron (Fe) availability is scarce. Therefore, experimental comparison of soil requirements (acidic Podzol vs alkaline Rendzina) of these species was carried out. The study was designed as a pot experiment and conducted under field conditions. Fe availability was increased by application of Fe-HBED. Reactions of plants to edaphic conditions were determined using growth measurements, leaf morphometric measurements, chlorosis scoring, chlorophyll content and chlorophyll *a* fluorescence (OJIP) quantification as well as determination of element content (Ca, Mg, Fe, Mn, Zn and Cu). Growth and leaf morphometrical traits of the studied congeneric species were affected similarly by the soil type and differently by the chelate treatment. Increased availability of Fe in Rendzina contrasted the species, as treatment with 25 µmol Fe-HBED kg^{-1} soil promoted growth only in *C. stoebe*. Both species turned out to be resistant to Fe-dependent chlorosis which was also reflected in only minor changes in chlorophyll *a* fluorescence parameters. Both species showed relatively low nutritional demands. Surprisingly, Fe-HBED did not stimulate Fe acquisition in the studied species, nor its translocation along the root:shoot axis. Furthermore, contrary to expectations, *C. scabiosa* took up less Fe from the acidic than alkaline soil. *C. scabiosa* not only absorbed more Ca and Zn but also translocated greater amounts of these elements to shoots than *C. stoebe*. Both species acquired more Mg on Podzol than on Rendzina which suggests adaptation allowing avoidance of aluminum (Al) toxicity on acidic soils. Overall, it seems that *C. scabiosa* prefers alkaline soils, whilst *C. stoebe* prefers acidic ones.

Subjects Ecology, Plant Science, Soil Science, Biosphere Interactions
Keywords *Centaurea*, Iron, Xerothermic grasslands, Congeneric species, Plant nutrition

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INTRODUCTION

The genus *Centaurea* L. belongs to the dicotyledonous Asteraceae family and is believed to be among the largest and the most taxonomically-challenging genera among Asteraceae (*Greuter et al., 2001*; *Aksoy et al., 2016*). Up to date it is known that *Centaurea* includes several hundreds of species (c.a. 400–700; *Greuter et al., 2001*), divided into three subgenera, *Centaurea sensu stricto*, *Lopholoma* (Cass.) Dobrocz. and *Cyanus* (Mill.) Hayek (*Hilpold et al., 2014*). All species within *Centaurea* are annual to perennial herbs (only rarely dwarf shrubs; *Dostál, 1976*), distributed naturally in Eurasia. Species of *Centaurea* occupy widely different open habitats varying in soil type, from inland sand dunes to meadows and from acidic to calcareous grasslands. Habitat preferences for vegetative growth of well-recognized species of *Centaurea* were widely described in the past (*Ellenberg, 1991*). Some species of *Centaurea* (*e.g.*, *C. diffusa* Lam., *C. jacea* L. and *C. stoebe* Tausch) were introduced to non-native areas (*e.g.*, North America), where they became weed and/or invasive species (*DiTomaso, 2000*; *Hahn, Buckley & Müller-Schärer, 2012*).

Grasslands are non-woody plant communities where edaphic characteristics play crucial role in formation and maintenance of their structure and floristic composition (*Puerto & Rico, 1997*; *Michaud et al., 2012*). The mosaic structure of soils and diversified requirements of plant species contribute to species richness of grasslands (*Leuchner & Ellenberg, 2017*). Various types of grasslands (*e.g.*, acidophilous and basiphilous grasslands; *Bothe, 2015*) establish on different, sometimes extremely contrasting types of soils. Considering chemical characteristics, soil acidity (pH) is among the major factors shaping plant establishment and survival in natural and man-made habitats (*Haling et al., 2011*; *Borhannuddin Bhuyan et al., 2019*). Soils with extremely different pH (remarkably acidic or alkaline) differ in other physical-chemical traits, including, among others buffering capacity, water holding capacity and availability of elements (*Jentsch & Beyschlag, 2003*; *Bothe, 2015*; *Kabała, 2018*), including crucial nutrients (*Leuchner & Ellenberg, 2017*; *Lambers & Oliveira, 2019*; *Schulze et al., 2019*). Soil acidification increases availability of cations (aluminum–Al, copper–Cu, iron–Fe, manganese–Mn and zinc–Zn, but not magnesium–Mg and calcium–Ca) due to desorption of elements from soil particles and their dissolution from minerals (*Lambers & Oliveira, 2019*). Thus, acidic soils provide sufficient amounts of some elements (most notably Fe and Mn) for plants, or even their availability may cause ion-specific toxicity (*e.g.*, Al; *Strawn, Bohn & O'Connor, 2020*). *Vice versa*, plants from alkaline soils encounter shortage of Fe and Mn and do not suffer from Al toxicity, as their solubility is controlled mainly by soil pH (*Bothe, 2015*). Moreover, alkaline soils are remarkably rich in freely available Ca, whilst sandy acidic soils are poor in this element unless influenced by human activity and marine or alluvial depositions. Availability of Zn and Cu can also be a limiting factor on both acidic and alkaline soils (*Lambers & Oliveira, 2019*). Additionally, concentration and speciation of nitrogen (NO_3^-/NH_4^+) and phosphorus ($HPO_4^{2-}/H_2PO_4^-$) strongly differs alkaline soils from acidic ones (*Leuchner & Ellenberg, 2017*).

Plant nutritional status is controlled by multilayer mechanisms acting simultaneously in roots and in shoots (*Baxter et al., 2008*). Although some cations (Ca, Mg and K) are

provided to plant cells mainly due to mass flow and diffusion, Fe, Mn, Zn and Cu are acquired by divalent cation transporters with a broad substrate ranges (*Korshunova et al., 1999*; *Lambers & Oliveira, 2019*). It implies that at least some micronutrients may compete for transporters and their acquisition by plants depends on local soil elemental ratio. Such mode of action was previously observed in in the case of increased availability of Fe (*e.g.*, due to application of chelates, including Fe-HBED) which is known to alter acquisition patterns of Mn and Zn and their allocation on a root-shoot axis (*Baxter et al., 2008*; *Wala et al., 2020*). As plant ionome (from cell to whole organism scale) is a complex networked structure (*Merchant, 2010*), its homeostasis can be disturbed by each element and reestablished by plant response if tolerance buffer is not exceeded. However, pH-dependent ion-specific limitations and co-limitations for plant growth are not well understood yet, especially in the case of habitat-specialized wild-living plants.

Iron (Fe) is a relatively common element and can be found in considerable amounts in various types of soils (*Colombo et al., 2014*). However, numerous biogeochemical processes lead to formation of Fe fractions that are poorly available for plants (Colombo et al., 2013). Such a situation prompted evolution of Fe acquisition mechanisms in terrestrial plants. Dicotyledonous plant species (including species of *Centaurea*) utilize two-step mechanism based on (1) acidification of rhizosphere by H^+ extrusion and (2) enzymatic reduction of Fe^{3+} to Fe^{2+} and uptake of the reduced Fe form (Strategy I mechanism; *Jeong & Connolly, 2009*; *Krohling et al., 2016*). Additionally, some Strategy I species secrete secondary metabolites (phytosiderophores) chelating Fe (*Sisó-Terraza et al., 2016*), which greatly helps plants occurring on alkaline soils (due to high soil buffering capacity halting acidification-dependent solubilization of Fe-containing compounds; *Grillet & Schmidt, 2017*). Disturbances in Fe scavenging process lead to nutritional deficiencies and, in severe cases, to development of chlorosis (*Celletti et al., 2016*). This, in turn, strongly limits plant primary functioning (*e.g.*, photosynthesis and acquisition of nutrients; *Venturas et al., 2014*; *Luna et al., 2018*). As soil acidity is the factor significantly shaping availability of Fe (*Bothe, 2015*), plants occurring on soils with extremely different pH values seem to have efficient adaptations regulating acquisition of this element or wide tolerance to sub- and supraoptimal Fe availability.

Distribution of plant species in wide range of conditions as well as their ability to adjust reactions to changing environment result from evolutionary conserved adaptations (*Schulze et al., 2019*). Even closely related plant species can show both extremely different (vicarious plant species; *Bothe, 2015*) or exceptionally similar requirements for habitat (congeneric plant species; *Silvertown & Wilkin, 1983*). Although congeneric species (including some species of *Centaurea*) occupy the same geographical areas and co-occur in plant communities, their abilities to win a competition for resources differ due to their limitations of growth and reproduction adjustments (*Gerlach & Rice, 2003*; *Eckhart et al., 2017*) partially resulting from differences in their ability to satisfy nutritional demands. For example, such a situation is hypothetically possible for congeneric plant species co-occurring in extremely contrasting communities, namely pioneer grasslands settled on acidic and dry soils (*Koelerio-Corynephoretea* Klika in Klika et Novák 1941 class) and xerothermic grasslands established on alkaline substratum (*Festuco-Brometea* Br.-Bl. et

Tüxen ex Soó 1947 class). However, it is not clear how species characterized with very wide tolerance to soil pH are able to persist and reproduce under such different conditions.

Comparisons of the soil requirements of plant species can give a valuable information about crucial determinants limiting their occurrence and co-occurrence (*Soliveres et al., 2014*). It can be hypothesized that congeneric plant species are expected to show some differentiation in resource utilization patterns or they occupy different microhabitats within the same type of community (*e.g.*, differing in soil acidity or availability of nutrients). However, studies on congeneric plant species regarding their pH-requirements are relatively rare—there is still lack of studies focusing even on common, wild species from the temperate zone. It is also not known if these species have truly similar nutritional requirements. Therefore, our intention was to draw an ecophysiological comparison between two widely-distributed and closely-related plant species, *C. scabiosa* and *C. stoebe* (Table 1), in order to estimate their habitat preferences. It is also not known if those species are chlorotic-prone and suffer from Fe limitations (both caused by Fe starvation and over-supplementation). Thus, we checked if soils differing in their characteristics (mainly acidity) as well as availability of Fe in alkaline soil, influence performance of the selected plant species. It was intended to test the following hypotheses: (1) *C. scabiosa* and *C. stoebe* have different preferences for soil type, (2) *C. stoebe* as an invasive species is better adapted to grow in contrasting soils than *C. scabiosa*, (3) availability of Fe influences functioning of the studied species. Thus, the following questions were raised: (1) Are the studied species similar in their soil requirements? (2) Is *C. stoebe* a superior species in terms of performance on contrasting soils? (3) Is Fe and element shaping growth of the studied species?

MATERIALS & METHODS

Description of the studied species

Centaurea scabiosa L. (abbreviated as Csc) is a perennial plant. Its root is well-developed and its stem is 30 to 150 cm high, woody, upright, angular (with rough edges) and branched from the middle (*Hegi, 1954*). Leaves are rough on abaxial side (rarely smooth), dark-green and pinnate with lanceolate sections. Inflorescences are settled individually at the tip of the branches. Flowers are purple, sometimes pink or white; the marginal ones are greatly enlarged and radiant, only rarely absent. It occurs on meadows, dry places, roadsides, bushy and scree slopes and in light woods, sometimes on rocks in mountainous areas (*Hegi, 1954*). *C. scabiosa* occurs naturally throughout Europe (except most southern and northern regions) and in Asia (up to Lake Baikal in Siberia; *Hegi, 1954*).

Centaurea stoebe Tausch (syn. *C. rhenana* Borheau; abbreviated as Cst) is a biennial to perennial plant. Its root is long, thick and woody and its stem is 20 to 90 cm high, stiffly upright, angular (rough at the edges) and branched in the middle. Its leaves are gray-green to almost green; the lowest leaves are pinnate, the following ones are pinnate and lobed, and the top ones are undivided and lanceolate. Inflorescences are very numerous (up to 200), settled individually at the tip of the branches. Flowers are bluish pink to pale pink, rarely white; the marginal ones are enlarged radiant. It occurs on sunny, grassy and rocky slopes, railway embankments, roadsides, vineyard edges and ruderal

Table 1 Interspecific differences in reaction to soil conditions of studied species of *Centaurea* and results of two-way ANOVA showing effects of species, treatment and their interaction on the measured traits.

Parameter	Relative difference between species (Csc:Cst ratio) and significance				*F* value and significance		
	p	r	r5	r25	(S) Species[a] df = 1	(T) Treatment[b] df = 3	S × T df = 3
Root FW	1.57 n.s.	2.34 n.s.	1.18 n.s.	0.78 n.s.	4.98*	1.78 n.s.	2.78 n.s.
Shoot FW	0.93 n.s.	1.13 n.s.	0.82 n.s.	0.40*	4.70*	0.45 n.s.	3.24*
Root DW	1.78 n.s.	3.21*	1.63 n.s.	1.01 n.s.	16.44***	0.92 n.s.	2.32 n.s.
Shoot DW	0.91 n.s.	1.14 n.s.	0.84 n.s.	0.41 n.s.	4.17 n.s.	0.30 n.s.	2.93 n.s.
S:R FW ratio	0.53 n.s.	0.51*	0.75 n.s.	0.50 n.s.	32.35***	2.14 n.s.	1.22 n.s.
S:R DW ratio	0.44 n.s.	0.37***	0.55 n.s.	0.42*	60.51***	2.35 n.s.	1.39 n.s.
Leaf FW	1.97***	2.47***	2.02***	1.34 n.s.	132.01***	3.65*	4.78**
Leaf DW	1.74**	2.29***	1.98***	1.20 n.s.	93.78***	1.74 n.s.	5.68**
Number of leaves	0.59*	0.58 n.s.	0.56 n.s.	0.35***	66.95***	1.90 n.s.	1.50 n.s.
Leaf area	1.70**	2.04***	1.64***	1.24 n.s.	78.58***	5.10**	2.95*
LDMC	0.89 n.s.	0.92 n.s.	0.98 n.s.	0.92 n.s.	11.09**	1.45 n.s.	0.91 n.s.
SLA	0.99 n.s.	0.89 n.s.	0.82*	1.02 n.s.	6.51*	1.66 n.s.	3.14*
IDC score[c]	1.00 n.s.	1.00 n.s.	1.00 n.s.	1.00 n.s.	1.00–	1.00–	1.00–
Chlorophyll content	0.94 n.s.	0.98 n.s.	1.00 n.s.	0.95 n.s.	2.95 n.s.	0.20 n.s.	0.67 n.s.
N	0.85 n.s.	0.92 n.s.	1.00 n.s.	0.96 n.s.	5.43*	4.48**	1.48 n.s.
F_V/F_M	1.02 n.s.	1.00 n.s.	1.01 n.s.	1.00 n.s.	3.20 n.s.	6.60***	1.40 n.s.
F_0	0.99 n.s.	1.02 n.s.	0.95 n.s.	0.99 n.s.	0.55 n.s.	0.25 n.s.	0.53 n.s.
F_J	1.00 n.s.	0.96 n.s.	0.88 n.s.	0.97 n.s.	6.10*	0.55 n.s.	1.60 n.s.
F_I	1.10 n.s.	1.02 n.s.	0.94 n.s.	0.93 n.s.	0.04 n.s.	1.28 n.s.	2.57 n.s.
F_M	1.06 n.s.	1.02 n.s.	0.97 n.s.	0.98 n.s.	0.19 n.s.	1.51 n.s.	0.98 n.s.
F_V	1.08 n.s.	1.02 n.s.	0.97 n.s.	0.98 n.s.	0.49 n.s.	2.36 n.s.	1.15 n.s.
S_M	0.93 n.s.	0.92 n.s.	1.03 n.s.	0.95 n.s.	2.92 n.s.	5.79**	0.75 n.s.
Area	1.01 n.s.	0.95 n.s.	1.00 n.s.	0.94 n.s.	0.56 n.s.	7.09***	0.27 n.s.
M_0	0.85 n.s.	0.91 n.s.	0.84*	0.98 n.s.	19.32***	5.05**	1.72 n.s.
V_I	1.05 n.s.	1.00 n.s.	0.96 n.s.	0.92*	2.64 n.s.	3.34*	7.08***
V_J	0.93 n.s.	0.91 n.s.	0.86*	0.97 n.s.	15.61***	4.79**	1.34 n.s.
ABS/RC	0.90*	1.00 n.s.	0.97 n.s.	1.01 n.s.	4.93*	1.93 n.s.	3.51*
TR_0/RC	0.91*	1.00 n.s.	0.98 n.s.	1.01 n.s.	4.09*	1.05 n.s.	3.32*
ET_0/RC	0.96 n.s.	1.07 n.s.	1.10 n.s.	1.04 n.s.	3.50 n.s.	1.69 n.s.	2.12 n.s.
DI_0/RC	0.84*	0.99 n.s.	0.95 n.s.	1.01 n.s.	5.09*	4.43**	2.87*
ϕE_0	1.07 n.s.	1.07 n.s.	1.13 n.s.	1.03 n.s.	14.34***	5.63**	1.05 n.s.
PI_{ABS}	1.37 n.s.	1.18 n.s.	1.35 n.s.	1.05 n.s.	13.80***	6.45***	1.43 n.s.
Root Ca	1.55 n.s.	0.79 n.s.	0.74 n.s.	0.56 n.s.	4.44*	11.26***	1.68 n.s.
Root Mg	1.13 n.s.	0.61 n.s.	0.74 n.s.	0.58*	16.60***	7.26**	4.86**
Root Fe	1.10 n.s.	0.59 n.s.	0.54 n.s.	0.70 n.s.	9.82**	10.32***	1.65 n.s.
Root Mn	1.08 n.s.	0.78 n.s.	0.73 n.s.	1.27 n.s.	0.26 n.s.	5.44**	0.89 n.s.
Root Zn	1.14 n.s.	0.78 n.s.	0.66 n.s.	0.82 n.s.	8.82**	3.85*	2.82 n.s.
Root Cu	1.04 n.s.	0.72 n.s.	0.71 n.s.	0.87 n.s.	3.75 n.s.	0.56 n.s.	0.84 n.s.
Shoot Ca	2.17***	1.91***	1.96***	1.81***	173.15***	2.86 n.s.	0.37 n.s.

(Continued)

Table 1 (continued)

Parameter	Relative difference between species (Csc:Cst ratio) and significance				*F* value and significance		
	p	r	r5	r25	(S) Species[a] df = 1	(T) Treatment[b] df = 3	S × T df = 3
Shoot Mg	1.95***	1.15 n.s.	0.98 n.s.	0.89 n.s.	6.63*	21.87***	7.00**
Shoot Fe	1.88 n.s.	1.07 n.s.	1.34 n.s.	1.23 n.s.	7.39*	4.54*	0.62 n.s.
Shoot Mn	1.14 n.s.	1.39 n.s.	1.68 n.s.	1.38 n.s.	7.86**	2.09 n.s.	0.58 n.s.
Shoot Zn	3.07**	2.60*	3.28**	2.45 n.s.	62.64***	0.62 n.s.	0.59 n.s.
Shoot Cu	0.83 n.s.	1.07 n.s.	0.62 n.s.	1.13 n.s.	0.71 n.s.	0.28 n.s.	1.17 n.s.
SAP Ca	1.01 n.s.	1.07 n.s.	1.11**	1.12**	41.12***	12.06***	3.48*
SAP Mg	1.21 n.s.	1.30 n.s.	1.16 n.s.	1.19 n.s.	20.77***	7.82***	0.43 n.s.
SAP Fe	1.34 n.s.	1.49 n.s.	1.79 n.s.	1.47 n.s.	24.73***	7.36**	0.11 n.s.
SAP Mn	1.00 n.s.	1.34 n.s.	1.44 n.s.	1.10 n.s.	10.12**	18.44***	2.12 n.s.
SAP Zn	1.48**	1.76***	2.13***	1.73***	172.21***	1.30 n.s.	2.41 n.s.
SAP Cu	0.90 n.s.	1.22 n.s.	0.96 n.s.	1.13 n.s.	0.71 n.s.	1.94 n.s.	1.44 n.s.
Root Fe:Mn ratio	1.00 n.s.	0.81 n.s.	0.76 n.s.	0.60 n.s.	11.10**	2.44 n.s.	2.12 n.s.
Shoot Fe:Mn ratio	1.70 n.s.	0.81 n.s.	0.85 n.s.	0.88 n.s.	0.39 n.s.	7.27**	1.67 n.s.

Notes:

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.005$.
n.s. – not significant.
[a] *C. scabiosa* or *C. stoebe*.
[b] Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 µmol Fe-HBED kg^{-1} soil (r25).
[c] Due to no variance of this trait, ANOVA analysis was not conducted.
Each value is a ratio of means of a given parameter measured in Csc and Cst. The values >1 detect that trait dominate in *C. scabiosa*, whereas values <1 indicate the opposite. Differences between mean values of each parameter were checked using two-way ANOVA (followed by Bonferroni's post-hoc test; n = 4–16, depending on parameter; for specific information see main text of the article and figure captions).

sites (*Hegi, 1954*). *C. stoebe* occurs naturally in Western, Southern (except Iberian Peninsula) and Eastern Europe (up to Caucasus; *Hegi, 1954*).

According to *Ellenberg (1991)*, both species have very similar centers of abundance. They can be found on well-lit places, on dry and extremely dry soils that are rather infertile (Table S1). According to this author the differences between the studied species are rather slight, however, *C. scabiosa* prefers slightly wetter and more fertile soils than *C. stoebe* (Table S1). The available data indicate that *C. scabiosa* and *C. stoebe* occur in both alkaline xerothermic and dry acidic grasslands, however their requirements for soil acidity are not clear (*Hegi, 1954*; *Ellenberg, 1991*; *Czyżewska, 1992*; *Matuszkiewicz, 2001*; *Kącki, Czarniecka & Swacha, 2013*, as well as personal observations of M. Wala and J. Kołodziejek).

Properties of soils, experimental setup and growth conditions

The soils used in this study were as those used in our previous investigation (for full spectrum of physical-chemical properties of the used soils see *Wala et al., 2020*). Entic Podzol (hereafter referred to as Podzol) characterized with low pH (pH_{KCl} = 4.3), low nutrient content (0.021% N, 56 mg P kg^{-1} soil) and high content of available

Fe (503 mg kg^{-1} soil). Rendzic Leptosol (hereafter referred to as Rendzina) was characteristic of higher pH (pH_{KCl} = 7.3), higher nutritional quality (0.220% N, 46 mg P kg^{-1} soil) and lesser content of available Fe (414 mg kg^{-1} soil) than Podzol.

Seeds of *C. scabiosa* and *C. stoebe* were collected at the maturity stage in 2018 from at least 30 plants from single, representative populations grown under optimal conditions in the Didactic-Experimental Garden of the Faculty of Biology and Environmental Protection (University of Lodz), central Poland (51°78′N; 19°48′E). Subsequently, the seeds were stored in paper bags for two weeks in constant laboratory conditions (21 °C; low humidity) and all damaged, discolored and malformed ones were discarded. Then they were stratified at 5 °C for 8 weeks before being used in the experiment.

The seeds of each species were mixed before the sowing for randomization. The seedlings were established from cold-stratified seeds in garden trays in Podzol or Rendzina and then randomly-selected ones from each soil were transplanted to 1.5 dm^3 pots filled with Podzol or Rendzina, respectively. The plants were grown under field conditions (full sunlight) in the Didactic-Experimental Garden of the Faculty of Biology and Environmental Protection (University of Lodz). Climate of this area is temperate. The seasons are clearly differentiated and typical of the temperate zone. Weather conditions of the experimental period (1st April to 1st October 2019; Fig. S1) were given according to the measurements of the Institute of Meteorology and Water Management – National Research Institute (Warszawa, Poland). The average lowest temperature during experiment was 10.1 °C (April), and the average highest temperature was 22.2 °C (June). Total precipitation during the experiment was 210.4 mm with the maximum in September and the minimum in June. The plants were grown in soil with optimal moisture (maintained with water used for the preparation of the tested Fe-HBED solutions). The plants were hand-weeded when any emergence of other plants from soil seed bank was observed.

Fe-HBED chelate (N,N′-di(2-hydroxybenzyl)ethylenediamine-N,N′-diacetic acid iron (III) sodium salt; 7% Fe; PPC ADOB, Poland) was selected as Fe carrier due to its outstanding ability to deliver Fe to plants under alkaline conditions (the process simulates Fe chelation in natural conditions; *López-Rayo, Hernández & Lucena, 2009*). Doses used in this study were as the same as those used in our previous investigation (*Wala et al., 2020*), because they allow to study individual Fe requirements of a given species (*Venturas et al., 2014*). After acclimatization (one week), the plants grown on Rendzina were randomly grouped and exposed to 0 (r), 5 (r5) or 25 (r25) μmol Fe-HBED kg^{-1} soil (prepared using tap water). The Podzol-grown plants (p) were treated solely only with the same volume of water without addiction of Fe-HBED (as same as the plants from Rendzina without addition of Fe-HBED). The plants were supplied with selected solutions every two weeks (5 doses in total). The experiment was set up on 1st April (seed sowing) and ended on 1st October (the day of measurements and collection of plant material). All further measurements were done on the same set of plants (four plants per treatment were used; n = 4).

Measurement of growth-related traits

Growth of the studied plant species was measured in order to estimate their preferences for edaphic conditions and to detect if they encounter any Fe-specific limitations on the alkaline soil. Fresh (FW) and dry weights (DW) of roots and shoots were determined by weighting, drying and re-weighting of the plant material after storage at 60 °C (c.a. 48 h). Partitioning of FW and DW between roots and shoots was estimated as a shoot:root (S:R) ratio. Leaf morphometrical analysis was performed in order to estimate resource utilization under different edaphic conditions. All measurements pertaining to leaf traits were conducted before determination of plant weight. The leaves were counted after extraction of plants from soil. Then, two (*C. scabiosa*) or four (*C. stoebe*) fully developed and representative leaves per plant were rapidly excised and weighted (FW) on an analytical balance (the number of assayed leaves per plant depended on the number of fully developed ones in both species). Subsequently, leaf area was measured using a CI-202 portable laser area meter (CID Bio-Science, USA). Then, the leaves were separately dried and re-weighted to obtain their DW. Specific leaf area (SLA) was calculated as the area per DW of a leaf blade (*Garnier et al., 2001*) and expressed in $cm^2\ mg^{-1}$. Leaf dry matter content (LDMC) was calculated as the amount of leaf DW per leaf FW and expressed in mg DW g^{-1} FW. All FW and DW measurements were taken using four plants per treatment and two (*C. scabiosa*) or four (*C. stoebe*) leaves per plant.

Chlorosis scoring and measurements of chlorophyll content

Measurements of chlorosis (both quantitative and qualitative) were conducted in order to detect soil-dependent limitations to biosynthesis and maintenance of photosynthetic pigments (signs of nutrient scarcity) and relations between these failures and the soil Fe status. The five-grade scale (*Wala et al., 2020*), adapted from the previous studies on Fe-dependent chlorosis (*Wang et al., 2008*), was used to visually estimate chlorosis (IDC score). Four plants per treatment were used for chlorosis scoring.

Chlorophyll content was determined using non-destructive fluorescence method utilizing a portable chlorophyll content meter, CCM-300 (Opti-Sciences Inc., Hudson, NH, USA) basing on findings presented by *Gitelson, Buschmann & Lichtenthaler (1999)*. Chlorophyll content was measured according to the standard protocol and calculated as mg m^{-2}. Chlorophyll content was measured using four fully developed leaves per plant and four plants per treatment.

Measurement of chlorophyll *a* fluorescence

Functioning of photosynthetic apparatus was inspected in order to detect if it is affected (improved or worsened) by edaphic conditions or availability of Fe on the alkaline soil. The polyphasic rise in chlorophyll *a* fluorescence (OJIP) transient was inspected with handheld PAM-type fluorometer, FluorPen FP100 (Photon System Instruments, the Czech Republic) basing on the findings of *Strasser, Tsimilli-Michael & Srivastava (2004)*. The records were gathered during optimal weather conditions (temperature > 18 °C, no cloud cover or rainfall). Prior to the data collection, the leaves were adapted in darkness for 20 min (using standard clips with shutters) in order to maximally diminish

energization-dependent fluorescence. All measurements were recorded using the standard protocol of the device. Selected parameters (Table S2) were automatically calculated by the pre-programmed equations (*Strasser, Tsimilli-Michael & Srivastava, 2004*). The OJIP parameters were recorded on two fully developed leaves (interveinal area) per plant and four plants per treatment. In order to show relative differences between the studied variants, fingerprints of the recorded parameters were presented using means of values normalized to the values measured on the plants grown on Rendzina (*Wala et al., 2020*) using standard spider plot technique (*Berger et al., 2007*).

Determination of elements and their partitioning

Quantification of elements and their partitioning was conducted in order to check nutritional status of plants and thus to estimate their requirements, tolerance to changing edaphic conditions and reaction to improved Fe availability. The elemental composition of roots and shoots was determined as previously described (*Wala et al., 2020*) using atomic absorption spectrometry (with SpectrAA 300 spectrometer; Varian Australia Pty. Ltd., Mulgrave, VIC, Australia). The content of each element was expressed in mg g^{-1} DW (Ca, Mg, Fe, Mn and Zn) or μg g^{-1} DW (Cu). Measurements were conducted on roots and shoots (leaves used to determine leaf parameters pooled with the remaining shoot material from a given plant) of four plants per treatment. In order to assess elemental allocation equilibria and to determine plant reactions to soil nutritional status, root-shoot transportation of each element was determined using shoot allocation percentage (SAP) proposed in the previous study (*Wala et al., 2020*). Ratio of Fe:Mn ratios in roots and shoots (a proxy of the mechanism contributing to avoidance of Fe-dependent limitations) were calculated using previously determined Fe and Mn contents.

Statistical analysis

Experiment was designed to tetraplicate each experimental variant for each species ($n = 4$). Normality of the data was examined with Kolmogorov–Smirnov's test and homogeneity of variances was examined with the Brown–Forsythe's test. The differences between treatments (p, r, r5 or r25) were detected by one-way ANOVA followed by Bonferroni's post-hoc test. Differences between variants were accepted as significant at $p < 0.05$.
To determine differences between the studied species, the plants from a given experimental variant were compared using two-way ANOVA followed by Bonferroni's post-hoc test (differences were accepted as significant at $p < 0.05$). Interspecific differences were presented as the ratios of a given trait value measured on *C. scabiosa* and *C. stoebe*. Thus, the values > 1 detect that trait dominate in *C. scabiosa*, whereas values < 1 indicate the opposite. Two-way ANOVA was conducted to detect effect of species and treatment and their interaction on the measured traits (differences were accepted as significant at $p < 0.05$). Correlation between Fe-HBED dose (r, r5 and r25 variants) and content of assayed elements was calculated separately for roots and shoots of both species using Pearson's correlation (differences were accepted as significant at $p < 0.05$). Principal Component Analysis (PCA) based on the correlation matrices was conducted using elemental datasets (from all tested variants, separately for roots and shoots) for generation

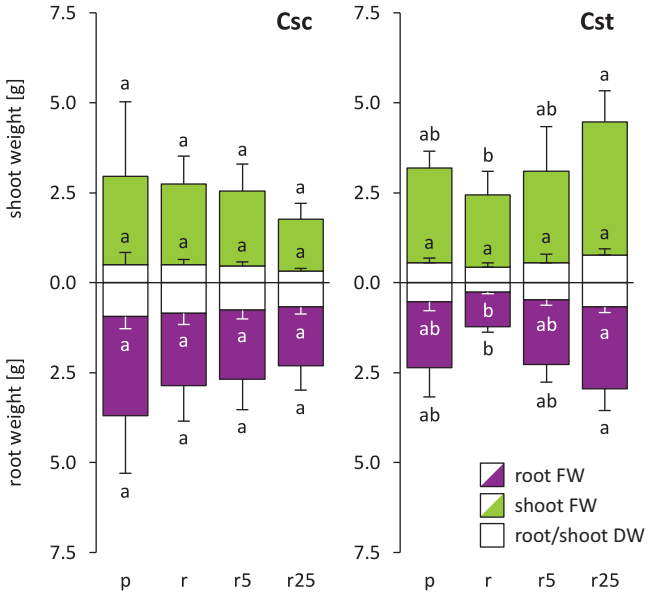

Figure 1 Growth of the studied species of *Centaurea* (measured as fresh weight – FW and dry weight – DW) in Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 µmol Fe-HBED kg^{-1} soil (r25). Values (mean ± SD) with different letters are significantly different between the treatments within a given species at $p < 0.05$ ($n = 4$; ANOVA with Bonferroni's post-hoc test). Whole bars (transparent + coloured area) – shoot/root FWs, transparent bars – shoot/root DWs.

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of principal components and respective values of FWs and DWs as additional variables (in order to estimate the influence of plant nutrition on growth of the studied species). All statistical analyses were conducted using Statistica™ v. 13.3 (Tibco Software Inc., Palo Alto, CA, USA).

RESULTS

Effects of edaphic conditions of growth

Soil type influenced neither FW nor DW of *C. scabiosa* and *C. stoebe*, however slight preferences towards acidic soil was observed in the latter (Fig. 1). Furthermore, Fe-HBED treatments did not alter weight of *C. scabiosa*, whilst increases in FW of shoots and roots as well as in DW of roots of *C. stoebe* were observed (Fig. 1). The latter parameter was increased by c.a. 150% after application of 25 µmol Fe-HBED kg^{-1} soil, comparing to the untreated Rendzina-grown plants. Although each species differentially allocated biomass, they responded very similarly to the tested conditions, as S:R ratios of DW and FW were not altered by treatments (Fig. S2). Considering interspecific differences, *C. scabiosa* had more developed roots when grown on untreated Rendzina and definitely smaller shoots on Rendzina supplied with 25 µmol Fe-HBED kg^{-1} soil than *C. stoebe* (Table 1). Furthermore, *C. scabiosa* allocated definitely more biomass into roots, which was reflected in FW and DW S:R ratios (Table 1). Almost all abovementioned measured growth parameters (except shoot DW) depended on species, whereas treatment and interaction of species and treatment had no significant effects (except shoot FW) (Table 1).

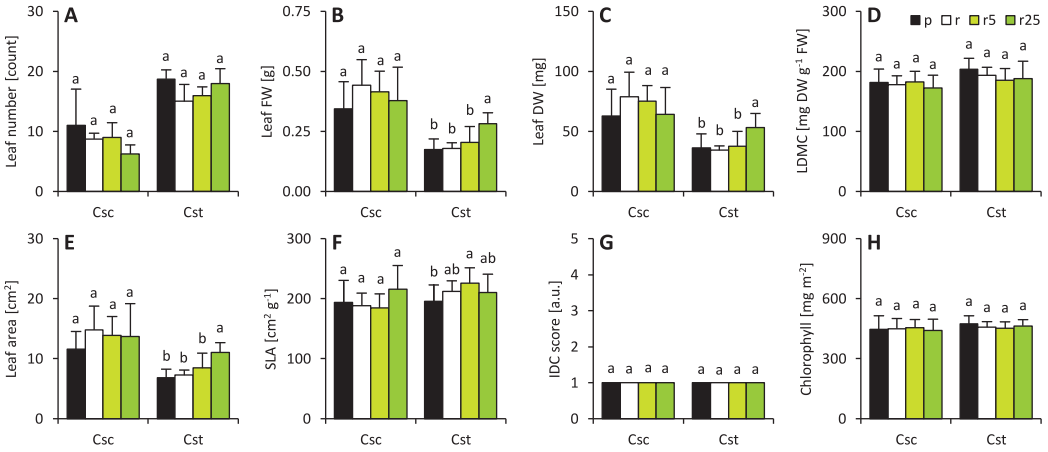

Figure 2 Characteristics of the leaves (A–number of leaves, B–leaf fresh weight–FW, C–leaf dry weight–DW, D–leaf dry matter content–LDMC, E–leaf area, F–specific leaf area–SLA, G–grade of iron dependent chlorosis–IDC score, H–chlorophyll). The IDC scores are as follows: 1–no visual signs of chlorosis (green leaf blades), 2–slight yellowing of leaves, 3–evident interveinal chlorosis (green veins and yellow interveinal areas), 4–interveinal chlorosis and slightly developing necrosis, 5–severe chlorosis (yellow veins and interveinal areas) and necrosis. Values (mean ± SD) with different letters for each parameter are significantly different between the treatments within a given species at $p < 0.05$ ($n = 8$ for Csc and $n = 16$ for Cst for leaf number, FW, DW, LDMC, area and SLA; $n = 4$ for IDC score and $n = 16$ for chlorophyll content; ANOVA with Bonferroni's post-hoc test).

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Soil type and Fe-HBED treatments did not affect leaf-associated traits in *C. scabiosa* (Fig. 2). On the other hand, 25 µmol Fe-HBED kg^{-1} soil increased leaf FW and DW in *C. stoebe* (Fig. 2). Furthermore, the greater the dose of Fe-HBED, the greater the leaf area of *C. stoebe* was observed (Fig. 2). Interestingly, treatment with 5 µmol Fe-HBED kg^{-1} soil caused the highest values of SLA in this species (Fig. 2). Moreover, the leaves of *C. scabiosa* were characterized with greater weight and area at the cost of their number, when compared to *C. stoebe* (Table 1). All measured leaf-associated traits were affected by species, whilst the treatment affected only leaf FW and area (Table 1). Several traits (leaf FW and DW, area, SLA) depended on the interaction between the studied factors (Table 1).

Resistance to Fe-dependent chlorosis and chlorophyll *a* fluorescence

Both species performed without any visual signs of chlorosis which was also reflected in qualitative IDC score and quantitative measurements of chlorophyll content (Fig. 2). Interspecific differences were not observed for these parameters (Table 1). It was reflected in two-way ANOVA analysis (Table 1).

Significant, treatment-dependent changes in OJIP parameters were only rarely observed. Podzol-grown *C. scabiosa* and *C. stoebe* showed similar performance of photosynthetic apparatus compared to the respective plants grown on Rendzina (Fig. 3). Addition of 5 µmol Fe-HBED kg^{-1} soil to Rendzina did not cause any changes in both studied species while that of 25 µmol Fe-HBED kg^{-1} soil affected only *C. scabiosa* (higher values of M_0 and lower of V_I) (Fig. 3) as compared to the untreated Rendzina. Most pronounced differences between the species were observed when they were grown on

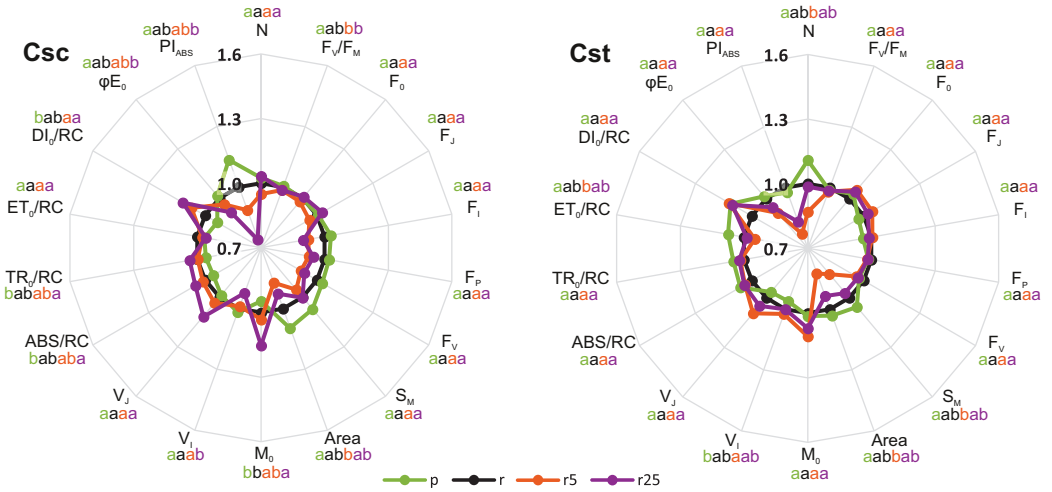

Figure 3 Relative changes of chlorophyll *a* fluorescence parameters (OJIP test) in leaves of the studied species of *Centaurea* grown on Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 µmol Fe-HBED kg^{-1} soil (r25). Relative values (means) were calculated as ratios between mean values measured on plants subjected to a given treatment and mean values measured on Rendzina-grown plants. Values with different letters are significantly different between the treatments within a given species at $p < 0.05$ ($n = 8$; ANOVA with Bonferroni's post-hoc test). The earlier letter indicates a significantly higher value of the parameter. The color of letter-based statistical indicators refers to the respective experimental variant as indicated in the legend. See Table S2 for definitions of plotted parameters. Full-size DOI: 10.7717/peerj.12417/fig-3

Podzol (ABS/RC, TR_0/RC, DI_0/RC, PI_{ABS}) (Table 1). Furthermore, lower values of M_0 and V_J were observed in Rendzina-grown *C. scabiosa* plants (supplied with 5 µmol Fe-HBED kg^{-1} soil) than in respective *C. stoebe* ones (Table 1). According to two-way ANOVA analysis, species significantly affected 9 out of 18 OJIP parameters, whereas treatment 10 out of 18 (Table 1). Only 4 out of 18 measured OJIP traits were affected by interaction between the studied factors (Table 1).

Elemental composition, allocation of elements, Fe:Mn ratios, influence of Fe-HBED on elemental composition and relation between nutritional status and growth

The soil type and supplementation with Fe-HBED altered the contents of several elements in roots of the studied species. That of Ca was not different considering plants grown on different soil types, however, treatment with Fe-HBED slightly increased it in both *C. scabiosa* (5 µmol Fe-HBED kg^{-1} soil) and *C. stoebe* (5 and 25 µmol Fe-HBED kg^{-1} soil) (Fig. 4). *C. scabiosa* acquired more Mg when grown on Podzol than when grown on Rendzina, whereas contents of this element were stable in *C. stoebe* among all tested variants (Fig. 4). Surprisingly, Fe content in roots of *C. scabiosa* was not affected by soil type and Fe-HBED treatment, whereas *C. stoebe* acquired more Fe after treatment with Fe-HBED when compared to the Podzol-grown plants (Fig. 4). Mn content in roots was similar in both species (Fig. 4). The roots of Rendzina-grown *C. stoebe* plants contained more Zn than those of the Podzol-grown ones, whereas in the roots of *C. scabiosa* its content was similar among all treatments (Fig. 4). Contents of Cu were

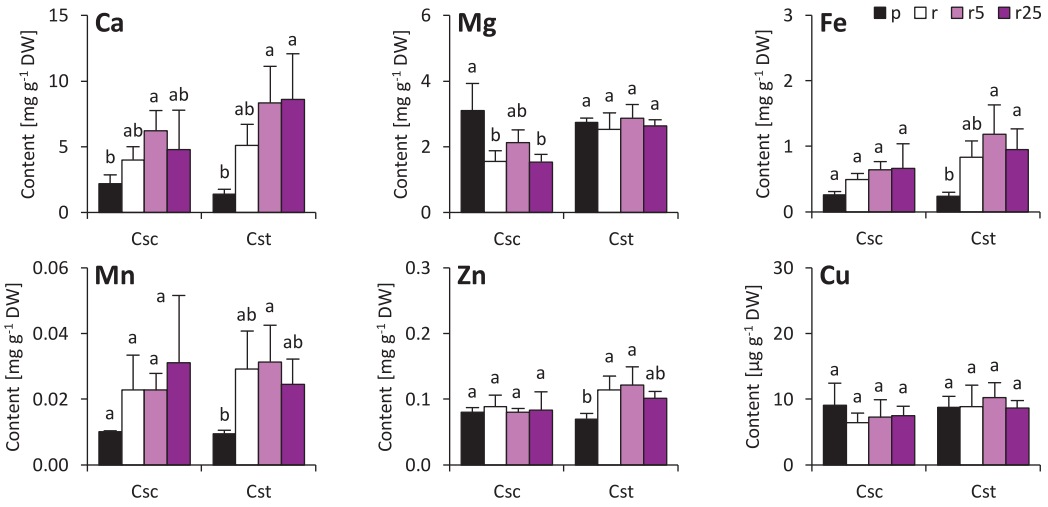

Figure 4 Root mineral composition (contents of calcium – Ca, magnesium–Mg, iron–Fe, manganese–Mn, zinc–Zn and copper–Cu) of the studied species of *Centaurea* grown in Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 µmol Fe-HBED kg. Values (mean ± SD) with different letters for each parameter are significantly different between the treatments within a given species at $p < 0.05$ ($n = 4$; ANOVA with Bonferroni's post-hoc test).

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stable in both studied species (Fig. 4). Comparing C. *scabiosa* with *C. stoebe* only lower contents of Mg (Rendzina-grown plants treated with the highest dose of Fe-HBED) was observed in the former species. (Table 1). Elemental composition of roots depended on species (except Mn and Cu) and treatment (except Cu) (Table 1). Interaction of these factors had effect only on Mg content (Table 1).

Minor changes were observed also in shoots of the studied species. Soil type and treatment with Fe-HBED did not influence contents of Ca, Mn, Zn and Cu in the shoots (Fig. 5). On the other hand, Fe content was greater in all Rendzina-grown *C. stoebe* plants than in Podzol-grown ones (treated as well as untreated with Fe-HBED; Fig. 5). Furthermore, both species showed higher Mg contents when grown on Podzol than on Rendzina (Fig. 5). It is worth noting that the shoots of *C. scabiosa* contained c.a. 100% more Ca and c.a. 150–200% more Zn than those of *C. stoebe*, whereas the contents of the other assayed elements were similar (except Mg in Podzol-grown plants; Table 1). Shoot contents of almost all elements (except Cu) affected by species, whilst only Mg and Fe depended on treatment (Table 1). Interactions between the studied factors were showed to influence only the content of Mg (Table 1).

Both studied species showed similar pattern of elemental allocation on the root:shoot axis, with important exceptions. They both allocated Ca in greater amount into shoots and Fe and Cu into roots (Fig. 6). However, it is worth noting that the plants grown on Rendzina supplied with Fe-HBED showed lesser Ca SAP values than those grown on Podzol (Fig. 6). Furthermore, in both species more Mg and Mn was allocated into shoots when the plants were grown on the acidic substratum than when grown on the alkaline soil (Fig. 6). Interestingly, only the allocation pattern of Zn contrasted the studied

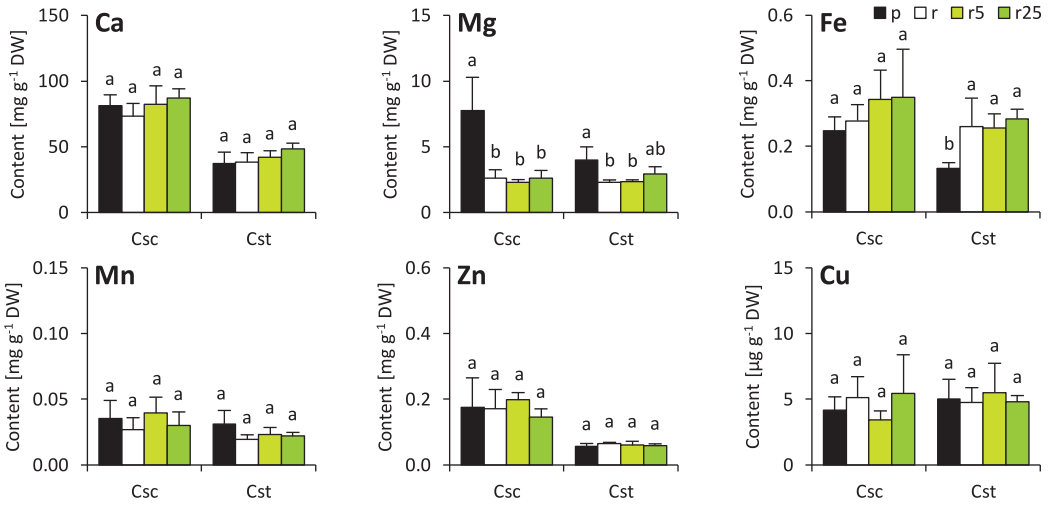

Figure 5 Shoot mineral composition (contents of calcium – Ca, magnesium–Mg, iron–Fe, manganese–Mn, zinc–Zn and copper–Cu) of the studied species of *Centaurea* grown in Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 µmol Fe-HBED k. Values (mean ± SD) with different letters for each parameter are significantly different between the treatments within a given species at $p < 0.05$ ($n = 4$; ANOVA with Bonferroni's post-hoc test).

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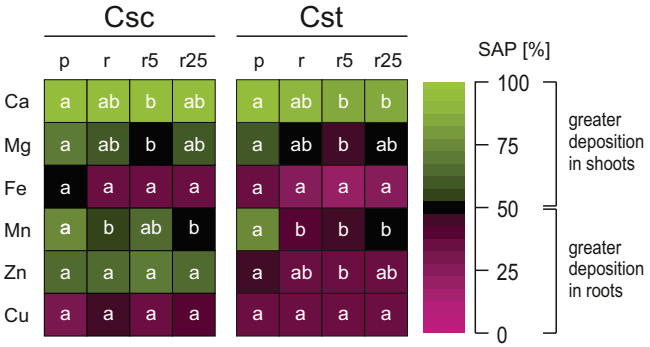

Figure 6 Heatmap of element partitioning measured as shoot allocation percentage (SAP) in the studied species of *Centaurea* grown on Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 µmol Fe-HBED kg^{-1} soil (r25). Values (means) with different letters for each parameter are significantly different between the treatments within a given species at $p < 0.05$ ($n = 4$; ANOVA with Bonferroni's post-hoc test). The earlier letter indicates significantly higher value of parameter.

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species, as *C. scabiosa* allocated this element mostly into shoots, in *C. stoebe* Zn remained in roots (Fig. 6).

Considering interspecific differences, *C. scabiosa* showed higher values of SAP than *C. stoebe*, including such elements as Ca, and Zn (Table 1). SAP values depended on species (excluding Cu) and treatment (excluding Zn and Cu) (Table 1). Only allocation of Ca was affected by interaction between the studied factors (Table 1).

Calculated Fe:Mn ratios (known to be reflection of plant reaction to iron deficiency) showed that Fe-HBED did not affect this parameter in either species (Fig. 7). Fe:Mn ratios in roots of the studied species were statically undifferentiated, however, slightly increasing

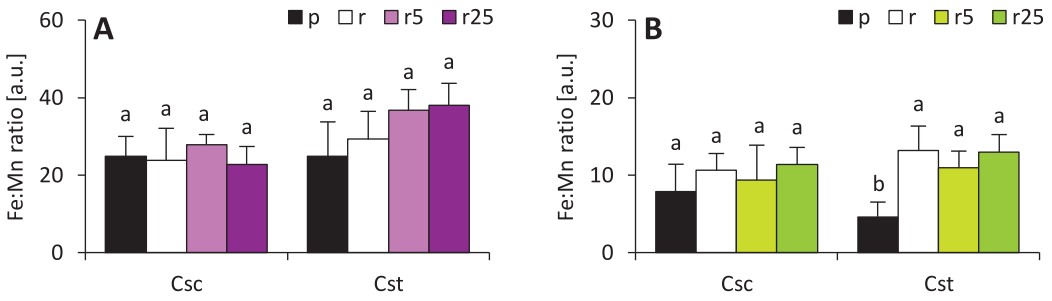

Figure 7 Iron to manganese ratio (Fe:Mn ratio) in roots (A) and shoots (B) of the studied species of *Centaurea* grown on Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 μmol Fe-HBED kg^{-1} soil (r25). Values (mean ± SD) with different letters for each parameter are significantly different between the treatments within a given species at $p < 0.05$ ($n = 4$; ANOVA with Bonferroni's post-hoc test). Full-size DOI: 10.7717/peerj.12417/fig-7

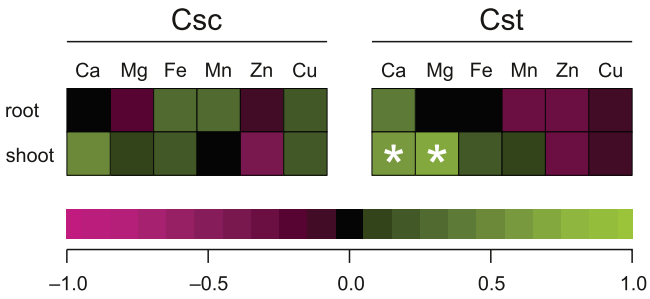

Figure 8 Spearman's rank correlation between dose of chelated Fe (0, 5 and 25 μmol Fe-HBED kg^{-1} soil) and content of the analysed elements in roots and shoots of the studied species of *Centaurea* grown on Rendzina. $^{*}p < 0.05$. Full-size DOI: 10.7717/peerj.12417/fig-8

trend in *C. stoebe* was observed (Fig. 7). The only significant difference was observed between shoots of Podzol- and Rendzina-grown *C. stoebe* plants (Fig. 7). Root and shoot Fe:Mn ratios in *C. scabiosa* and *C. stoebe* plants were not statistically different (Table 1). Root Fe:Mn ratio depended only on species, whereas shoot Fe:Mn ratio only on treatment (Table 1).

Significant correlation between the dose of Fe-HBED and the content of a given element was observed only in the case of Ca (R = 0.635) and Mg (R = 0.704) in shoots of *C. stoebe*, whilst all other calculated correlations were not significant (Fig. 8).

The PCA based on the data from elemental analysis showed that the first two principal components accounted for 72.14% (*C. scabiosa*) and 82.21% (*C. stoebe*) of the total variance in roots and 58.70% (*C. scabiosa*) and 65.03% (*C. stoebe*) of the total variance in shoots (Fig. 9). Considering loadings, Fe and Mn accounted the most for the first principal component in roots and Fe and Ca in shoots of *C. scabiosa* (Table S3). In the case of *C. stoebe*, Fe, Mn and Zn accounted the most for the first principal components in roots, whilst Mg and Mn in shoots (Table S3). Second principal components was associated with Zn (roots) and Mg (shoots) in *C. scabiosa*, whilst Cu loaded the most for second principal component in *C. stoebe* (Table S3). Considering third principal component, Mg (roots of both studied species) and Zn (shoots of both studied species) loaded the most (Table S3). The root and shoot FWs and DWs were positively correlated with the first

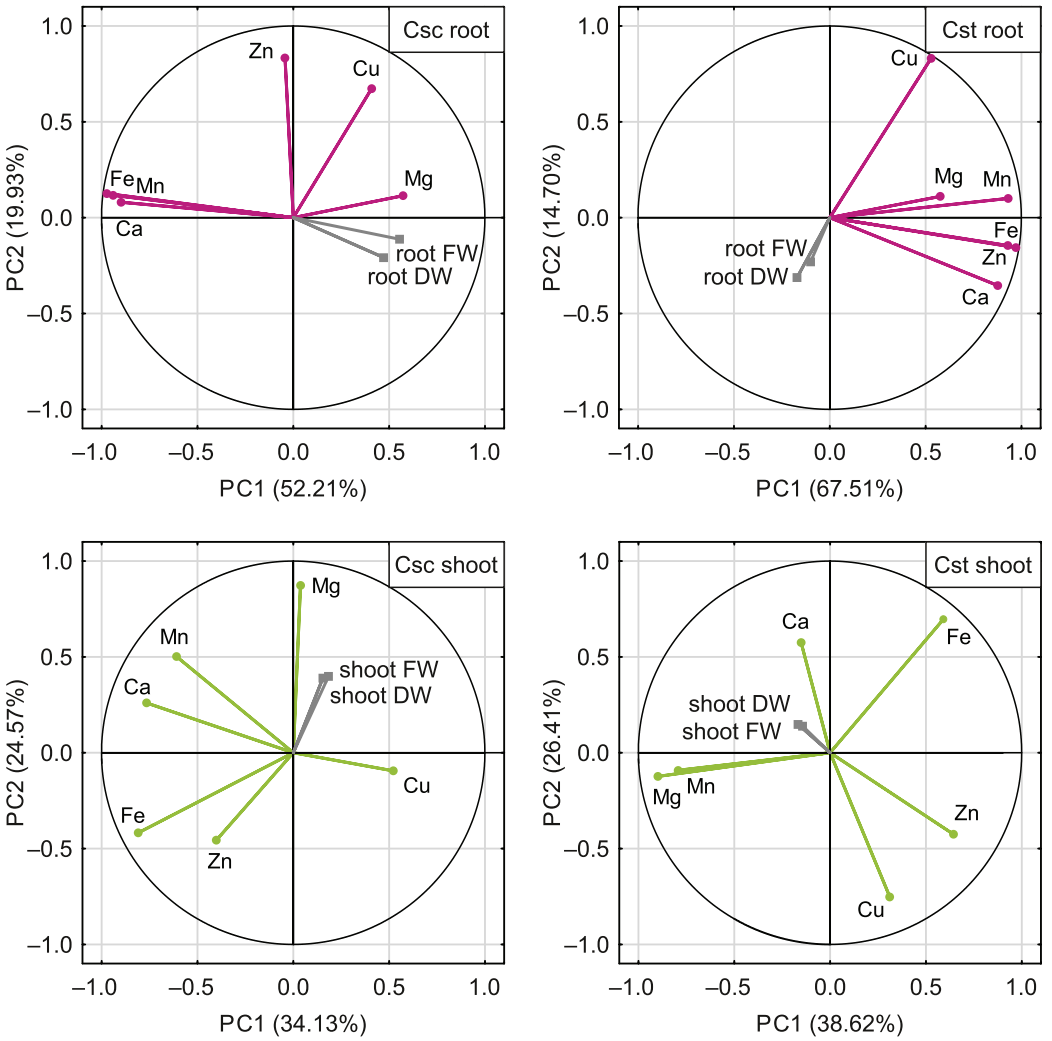

Figure 9 Correlation matrix of content of the analysed elements (Ca, Mg, Fe, Mn, Zn and Cu) and growth traits (root and shoot FW/DW) of the studied species of *Centaurea* plotted on two first principal components (PC) from Principal Component Analysis (PCA). Each point represents a variable, and each vector represents correlation between a given variable and PCs. Color-marked traits were used for generation of PC1 and PC2 (active variables) and grey-marked traits were plotted as additional variables. Percentages presented in parentheses indicate the explained variance. For details pertaining to the loadings of PC1, PC2 and PC3, see Table S3.

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principal component in *C. scabiosa*, whilst negatively in *C. stoebe* (Fig. 9). The values of root FWs and DWs in both species were negatively correlated with the second principal component, whilst the opposite was observed in the case of shoot FWs and DWs (Fig. 9).

DISCUSSION

Many terrestrial plant species suffer from Fe-dependent limitations (*Grime & Hutchinson, 1967*; *Sanz, Cavero & Abadía, 1992*; *Tagliavini & Rombolà, 2001*), however it seems not to be the case for the studied species of *Centaurea*. Development of Fe-dependent chlorosis is the best-known effect of severe Fe starvation

(*Lucena & Hernandez-Apaolaza, 2017*). The previous study using the same experimental setup revealed that some calcicole plant species from xerothermic grasslands (*e.g. Aster amellus* L, *Betonica officinalis* L. and *Prunella grandiflora* (L.) Scholler.; *Wala et al., 2020*) suffered from chlorosis due to Fe-dependent limitations. Visual signs of Fe deficiency were not observed in *C. scabiosa* and *C. stoebe* in the current study, which generally suggests that soil acidity and availability of Fe are not factors strongly limiting growth and development of these species (question 1, 2 and 3). To the best of our knowledge, this is the first experimental study investigating soil preferences of those species as well as in general, plant congeners with wide ecological amplitude in terms of soil pH.

The results indicated that there were no major obstacles for establishment of *C. scabiosa* and *C. stoebe* in totally contrasting soils, as there were no significant differences pertaining to growth between the plants grown on acidic Podzol and slightly alkaline Rendzina (question 1 and 2). Similar behavior can be observed in several other dicotyledonous plants (*e.g. Dianthus carthusianorum* L, *Euphorbia cyparissias* L. and *Hypericum perforatum* L.; *Stahevitch, Crompton & Wojtas, 1988*; *Krawczyk, Domagała-Świątkiewicz & Lis-Krzyścin, 2017*) that are able to persist in extremely different communities, namely acidic dry grasslands and alkaline xerothermic grasslands. It is also known that tetraploid, polycarpic *C. stoebe* subsp. *micranthos* became an invasive species. Although adaptive differences between diploids (studied *C. stoebe*) and tetraploids (*C. stoebe* subsp. *micranthos*) are not known, it can be hypothesized that the presented results partially explain why *C. stoebe* (especially *C. stoebe* subsp. *micranthos*) colonizes new geographical areas very efficiently (*Akin-Fajiye & Gurevitch, 2018*), taking also into consideration the fact that polyploids are better adapted to a wide spectrum of environmental conditions (*Te Beest et al., 2012*). It is in agreement with the studies showing that wide tolerance to edaphic conditions allows invasive behavior of other plant species (*Sanderson, Day & Antunes, 2015*; *Kołodziejek, 2019*). Considering growth of the studied species, is not fully clear why *C. scabiosa* does not show invasive behavior. This plant is known to be a 'stress-tolerant' subordinate species from alkaline grasslands (*Moora, Öpik & Zobel, 2004*). It is probable that other characteristics, *e.g.*, ability to cope with environmental stressors, pollination ecology, germination ecology and biotic interactions hinder efficient propagation of this species. Furthermore, considering reaction to soil type, interspecies differences in growth (*e.g.*, weight allocation pattern and leaf characteristics) are likely to result from physiognomy of the studied species, but not from their individual reaction to substratum (alkaline Rendzina *vs* acidic Podzol). To support this finding, it can be mentioned that the studied plant species showed different weight allocation between roots and shoots, but the general trend in a given species of S:R ratio remained unaffected. Although it is still an element of scientific debate, some studies (*e.g.*, *Funk, 2013*) suggested that high allocation of biomass into shoots was typical of invasive species (which is in agreement with the presented study considering this trait in *C. stoebe*, but not in *C. scabiosa*).

Another trait that is often assessed in studies on ability to cope with stress, competitiveness and invasiveness of plant species is SLA, as more invasive plants and

species with greater amplitude of tolerance to edaphic conditions are often characterized with higher values of this parameter (*Baruch & Goldstein, 1999*). Although differences between the studied species were lesser than one might expect, SLA values were significantly higher in *C. stoebe* than in *C. scabiosa* in one case (r5), which is in agreement with common beliefs about the connection between SLA and invasiveness. Moreover, this parameter is sometimes described as a trait connected with assimilation rate (*Storkey, 2005*), however, considering OJIP parameters measured in this investigation (*e.g.*, F_V/F_M and PI_{ABS}), it is not very probable in the case of the studied species. In general, the numbers of leaves in rosettes of both studied species were within ranges reported before (*Moora, Öpik & Zobel, 2004*; *Henery et al., 2010*). Greater number of leaves in *C. stoebe* than in *C. scabiosa* can be beneficial for the former species under the conditions provoking accelerated aging of leaves or their destruction due to environmental stress under harsh conditions of inland sand dunes (*e.g.* due to sandblasting caused by wind; *Maun, 1994*). In the case of *C. stoebe*, it can be also interpreted as a long-term investment in higher competitiveness (*Moora, Öpik & Zobel, 2004*). However, none of the tested variants influenced leaf traits in C. *scabiosa*. It is a relatively rare situation, as contrasting edaphic conditions and thus nutrient availability (including Fe) were showed to affect leaf lamina morphometric parameters (*McDonald et al., 2003*; *Masuda et al., 2018*; *Wala et al., 2020*). This supports the conception that *C. scabiosa* is in general species a with wide ecological amplitude (with regard to soil preferences), at least considering theoretical niche optima. Rendzina supplemented with 25 μmol kg^{-1} soil Fe-HBED promoted increase in leaf weight and area in *C. stoebe*, even comparing to the Podzol-grown plants. This suggests that growth and development of this plant on calcareous grasslands is at least partially dependent on interactions with neighboring plants and soil microbiota, both regulating availability of Fe (*Colombo et al., 2014*).

Looking through the prism of stable co-existence of congeneric species, at least minimal differences (*e.g.*, related to individual nutrition requirements) between them must occur (*Silvertown & Wilkin, 1983*). The major difference between the studied species consisted in their reactions to increasing availability of Fe on the alkaline soil. Application of Fe-HBED into Rendzina was beneficial only for *C. stoebe*, which was reflected in growth-related parameters. It suggests that availability of Fe can be a limiting factor for this species growing on an alkaline soil, because promotion of growth by addition of a given element is known to be a sign of a specific limitation. It suggests that the edaphic optimum of *C. stoebe* is associated with acidic soils (with high availability of Fe) or soils on which geochemical and biological processes promote solubilization of Fe-containing compounds. However, application of the chelate did not trigger any significant changes in the elemental status in shoots and roots of this species (including Fe content). It is possible that the improved growth after application of Fe-HBED resulted from altered availability and/or acquisition of other, non-assayed elements (*e.g.*, macro- and micronutrients or ballast elements; *Baxter et al., 2008*), which optimize nutritional status of plants. It is also possible, that increasing availability of Fe unburdened secondary metabolism due to Fe-availability-dependent stimuli (as there is a negative feedback loop

between iron availability and phytosiderophore release; *Ma et al., 2003*; *Chen, Wang & Yeh, 2017*). It is known that *C. stoebe* exudates (±)-catechin into rhizosphere (*Blair et al., 2006*). This flavonoid-type polyphenolic compound is classified as phytosiderophore due to its catechol moiety covering chelation of various metals, including Fe (*Chobot & Hadacek, 2010*). If exudation of (±)-catechin substantially contributes to Fe scavenging in *C. stoebe* under physiological conditions, carbon skeletons saved from siderophore biosynthesis pathway (due to increased availability of Fe) are likely to be redirected into other metabolic processes, including also those associated with plant growth *per se*. This explanation supports the conception that the primary role of (±)-catechin consists in metal chelation (*Blair et al., 2006*; *Chobot et al., 2009*). Unfortunately, nothing is known about root exudation patterns of *C. scabiosa*. However, the presented data clearly showed that Rendzina-grown *C. scabiosa* had higher contents of Fe, Mn and Zn in roots than the Podzol-grown plants, which suggests that induction of phytosiderophore biosynthesis is triggered by alkaline substratum in this species as well. Similar mode of action was reported also in a wide spectrum of calcicole and calcifuge plant species (*Ström, Olsson & Tyler, 1994*). On alkaline soil phytosiderophore-caused nutritional improvements are more probable than those associated with enhanced rhizospheral acidification, due to very high buffering capacity of soil carbonate buffer, making rhizospheral acidification tough (*Grillet & Schmidt, 2017*). However, it is worth noting that plentiful exudates are not produced by plants when they encounter outstanding availability of divalent ions (*e.g.*, on acidic soils).

Acquisition of Fe and Mn on acidic soil is an interesting aspect of microelemental balance in the studied species. The contents of these elements are definitely lower than in the plants grown on Rendzina and even lower than in some remarkably calcicole species grown on the same acidic and sandy soil (*Wala et al., 2020*). It indicates that minimal nutritional demands of the studied species of *Centaurea* are low. It also suggests that acquisition of several divalent ions from alkaline soils is phytosiderophore- and/or pH-dependent in these species. Furthermore, these plants were able to satisfy their Fe demands on alkaline soil, which coincided with increasing content of Mn in their tissues. It resulted in more or less stable Fe:Mn ratio, which implies that the studied species are adapted to tolerate increased Mn uptake as a side effect of Fe scavenging. It is worth noting that low Fe:Mn ratio is known to promote chlorosis (*Gama et al., 2016*; *Zamboni et al., 2017*; *Wala et al., 2020*). However, the studied species of *Centaurea* are able to operate without signs of chlorosis when this parameter is even lower than in chlorosis-resistant calcicole plant species from xerothermic grasslands, *e.g.*, *Salvia verticillata* L. or *Veronica teucrium* L. (*Wala et al., 2020*). Considering the molecular mechanism of this adaptation, acquisition and vacuolar storage of excessive Mn (allowing functioning of Fe scavenging system) is the most plausible one, as this mechanism is known to support optimal functioning of Fe uptake (*Eroglu et al., 2016*).

Testing of chlorophyll *a* fluorescence was showed by numerous studies to be a tool allowing diagnosis of physiological state of a plant (*Kalaji et al., 2016*; *Stirbet et al., 2018*). For example, several investigations showed that some of OJIP parameters could be used for discrimination between plants tolerant and intolerant to deficiency and/or excess of Fe

(*Luna et al., 2018*, *Wala et al., 2020*). Both studied species did not show any symptoms of Fe- and/or $CaCO_3$-dependent limitations concerning photosynthetic apparatus functioning that are known from other plants species, *e.g.*, *Vitis vinifera* L. (*Shahsavandi et al., 2020*). On the other hand, slightly decreased values of PI_{ABS}, ϕ_{E0} and F_V/F_M as well as increased values of DI_0/RC, ABS/RC and M_0 in *C. scabiosa* plants after application of 25 µmol Fe-HBED kg^{-1} soil suggest that this species might not tolerate high availability of Fe in alkaline soil. Similar mode of action was reported in sweet potato (*Ipomoea batatas* L.) plants oversupplied with Fe (*Adamski et al., 2011*). It probably has some ecological implications for the studied species of *Centaurea*. Several studies showed that graminoid plant species exudate large quantities of Fe chelators, which modulate availability of this element for other plant species, including dicotyledons (*Dai et al., 2019*). Thus, it is very likely, that grasses being an important element of xerothermic grasslands (*e.g.*, *Brachypodium pinnatum* L, whose dominance has adverse effects on plant diversity; *Bobbink & Willems, 1987*) negatively affect *C. scabiosa*, making it a subordinate species due to solubilization of Fe-containing soil compounds. In contrast, such a situation could be beneficial (or at least neutral) for *C. stoebe*.

Comparing other aspects of elemental composition of the studied species, two major differences between them can be observed – *C. scabiosa* acquired greater amounts of Ca and Zn than *C. stoebe* (although relations between the tested variants within a given species were similar). *C. scabiosa* allocated Ca and Zn into shoots and roots, respectively. It is in agreement with the studies showing that *C. scabiosa* stores excess of Ca in leaf trichomes (probably in an oxalated form; *De Silva, Hetherington & Mansfield, 1996*). Besides natural and seminatural types of communities considered in this article (alkaline xerothermic and acidic dry grasslands), anthropogenic calamine soils rich in Zn were showed as substrata supporting growth of *C. scabiosa* and *C. stoebe* (*Skubała, 2011*; *Pająk et al., 2018*). Considering this information and the data presented in this study (including acquisition and allocation of Zn on the root-shoot axis), it can be proposed that the studied species have different strategies allowing their persistence on alkaline soils with at least average content of Zn; *C. scabiosa* can be treated as a shoot Zn accumulator (due to allocation of this element into shoot), whereas *C. stoebe* avoids acquisition of this element and its translocation to shoots. Similar differentiation (*Brown et al., 1995*) was showed between a Zn accumulator (*Thlaspi caerulescens* J. Presl & C. Presl) and a plant tolerant to Zn (*Silene vulgaris* (Moench) Garcke). The difference pertaining to Zn acquisition explains also why *C. scabiosa* can be found predominantly on more or less basic soils (*e.g.*, Rendzinas), even those rich in Zn (*e.g.*, post-mining ones; *Pająk et al., 2018*).

Comparing the Rendzina- and Podzol-grown plants, accumulation of Mg in aboveground organs was observed in the latter ones. Lesser availability of Mg in alkaline soils is known to be a result of soil pH (*Senbayram et al., 2015*) and high availability of Ca (*Guo et al., 2016*). However, similar limitations were observed on remarkably acidic soils due to H^+ enrichment of soil solution (*Senbayram et al., 2015*). The presented results indicate that low soil pH, and thus high H^+ and Al^{3+} concentrations, were not an obstacle for Mg scavenging in the studied species of *Centaurea*. Furthermore, even the low

content of Mg recorded in the Rendzina-grown plants did not trigger any detectable decrease in chlorophyll content nor severe malfunctions of photosynthetic apparatus. Interestingly, negative coincidence between Fe and Mg contents, which was observed in the presented study, was previously shown in some species, (*e.g.*, *Ulmus laevis* Pall. and *U. minor* Mill.; *Venturas et al., 2014*). Although this phenomenon was described in the past (*Fageria, 2001*), it is not known if Mg and Fe antagonism results from cause-effect relation or it just occurs due to Ca enrichment of soil. Additionally, accumulation of Mg may have a protective role when Al availability in soil is relatively high (*Bose, Babourina & Rengel, 2011*), which suggests additional adaptation allowing those species persistence on remarkably acidic soils, *e.g.*, Podzols (where the pool of available Al is high, especially at pH lower than five; *Abedi, Bartelheimer & Poschlod, 2013*).

CONCLUSIONS

In conclusion, both species are able to survive and develop under extremely different edaphic conditions. Both of them are well adapted xeric plants. However, the presented investigation indicated that ecophysiological optimum of *C. scabiosa* was shifted to alkaline soils (due to its reaction to Fe-HBED application into Rendzina as well as acquisition of Ca and Zn), whilst soil preference of *C. stoebe* was tilted towards acidic soils (or other soils with increased availability of Fe; most notably due to its reaction to Fe-HBED and physiognomy). *C. scabiosa* and *C. stoebe* are species invulnerable to severe Fe-dependent malnutrition. Even more, they have wide tolerance to nutritional soil status, as no serious symptoms of limitations were observed in this study. It can be proposed that high tolerance to edaphic conditions and low requirements of those species contribute to their increasing geographical range and/or high ability to persist in tough habitats. Their physiological adaptations allow them to colonize new areas, even on sites where patchy pattern of soil makes spread of other species tough. It is also worth noting that both species are able to develop without any signs of Fe-dependent chlorosis when grown in separation from other species. It means that the tested species do not necessarily require the common good of mobilized Fe (*e.g.*, from activity of other plants) to survive. This gives them advantage over other species during establishment on vegetation gaps and loose vegetation sites (*e.g.*, psammophilous grasslands).

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Mateusz Wala conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Jeremi Kołodziejek conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Janusz Mazur performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Alicja Cienkowska performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

This raw data from all experiments including measurements of growth, chlorosis, chlorophyll fluorescence and elemental composition are available in the Supplemental File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.12417#supplemental-information.

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P–3

Materiał uzupełniający

(Supplementary material)

Supplemental Table 1. Ecological indicator values describing realized niche optima of the studied species of *Centaurea*. Ordinal scale (1-9) of Ellenberg's Indicator Values follows Ellenberg (1991). L, light requirements ranging from 7 to 8, where 7 indicates semi-lit conditions (c.a. 30% of relative illumination) and 8 indicates light conditions (c.a. 40% of relative illumination); T, temperature requirements of 7 indicating species preferring warm conditions (characteristic of North European Plain); K, continentiality requirements ranging 3 to 5, where 3 indicates atlantic/subatlantic conditions and 5 indicates subatlantic/subcontinental conditions; F, soil moisture requirements ranging 2 to 3, where 2 indicates dry and extremely dry soils and 3 indicates dry soils; R, soil pH requirements of 8 indicating average basic soils originating from limestones; N, nitrogen availability requirements ranging 3–4, where 3 indicates slightly fertile soils and 4 denotes slightly and intermediately fertile soils; 0 – indifferent behavior, wide amplitude or unequal behavior in different areas.

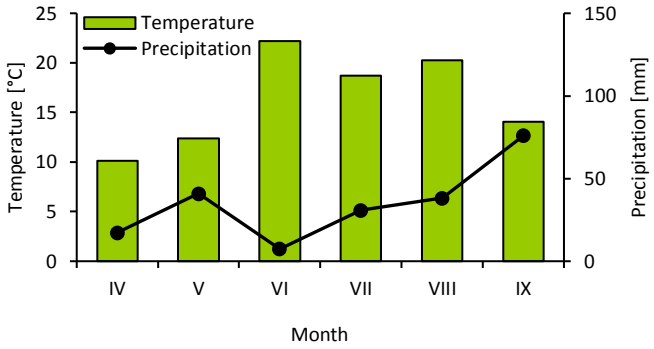
Name	Abbreviation	Ellenberg's Indicator Value					
		L	T	K	F	R	N
Centaurea scabiosa L.	Csc	7	0	3	3	8	4
Centaurea stoebe Tausch	Cst	8	7	5	2	8	3

Supplemental Table 2. Parameters derived from the OJIP transient used in this study, formulas of their calculation and definitions. List of studied parameters follows Wala et al., 2020.

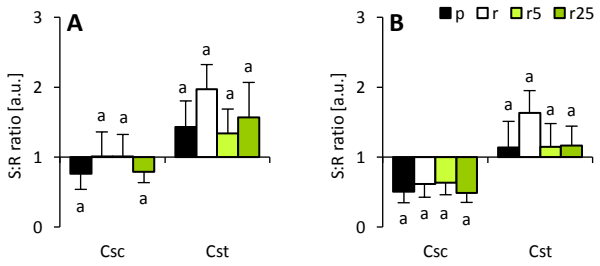
OJIP parameter	Formula	Definition
F_0 (=F_O)	F_0=F at 50μs (=F at O-step)	Fluorescence intensity at O-step (at 50 μs) (=Minimal fluorescence intensity)
F_J	F_J=F at 2ms (=F at J-step)	Fluorescence intensity at J-step (at 2 ms)
F_I	F_I=F at 60ms (=F at I-step)	Fluorescence intensity at I-step (at 60 ms)
F_M (=F_P)	F_M=F at 1s (=F at P-step)	Fluorescence intensity at P-step (at 1000 μs) (=Maximal fluorescence intensity)
F_V	$F_V=F_M–F_0$	Maximal variable fluorescence
V_J	$V_J=(F_J–F_0)/(F_M–F_0)$	Relative variable fluorescence at J-step (2 ms)
V_I	$V_I=(F_I–F_0)/(F_M–F_0)$	Relative variable fluorescence at I-step (60 ms)
F_V/F_M	-	Maximum quantum yield of primary PSII photochemistry
M_0	$M_0=TR_0/RC–ET_0/RC$	Approximated initial slope of the fluorescent transient
Area	-	Area between fluorescence curve and F_M (background subtracted)
S_M	S_M=Area/(F_M - F_0)	Standardized area above the fluorescence curve between F_0 and F_M
N	$N=S_M*M_0*(1/V_J)$	Number of Q_A redox turnovers until F_M is reached
φ_{E0}	$\varphi_{E0}=[1–(F_0/F_M)]*\psi_0$	Quantum yield for electron transport from Q_A to plastoquinone at t = 0
PI_{ABS}	$PI_{ABS}=\gamma RC/(1-\gamma RC)*\varphi_{P0}/(1-\varphi_{P0})*\psi_0/(1-\psi_0)$	Performance index of electron flux from PSII based to intersystem acceptors
ABS/RC	$ABS/RC=M_0*(1/V_J)*(1/\varphi_{P0})$	Photon flux absorbed by PSII antenna chlorophyll per RC at t = 0
TR_0/RC	$TR_0/RC=M_0*(1/V_J)$	Trapping flux leading to Q_A reduction per RC at t = 0
ET_0/RC	$ET_0/RC=M_0*(1/V_J)*\psi_0$	Electron transport flux per RC at t = 0
DI_0/RC	$DI_0/RC=(ABS/RC)–(TR_0/RC)$	Dissipated energy flux per RC at t = 0

Supplemental Table 3. Loading values of Principal Component Analysis (PCA) for the three first components (PC1, PC2 and PC3).

	Csc						Cst					
Variable	root			shoot			root			shoot		
	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3
Ca	0.257246	0.005445	0.140939	0.286050	0.045773	0.070116	0.189223	0.141798	0.005283	0.009848	0.208068	0.031800
Mg	0.104464	0.010976	0.575275	0.000698	0.517739	0.085120	0.081836	0.013928	0.872527	0.347898	0.009619	0.018384
Fe	0.302531	0.013303	0.023744	0.320113	0.118556	0.000615	0.233180	0.027611	0.010010	0.150875	0.305876	0.059267
Mn	0.281743	0.011454	0.007787	0.181540	0.171010	0.120975	0.214145	0.011485	0.091660	0.270671	0.005278	0.159999
Zn	0.000594	0.579445	0.168581	0.078565	0.140862	0.431413	0.212744	0.023883	0.000472	0.178825	0.114252	0.169807
Cu	0.053422	0.379377	0.083670	0.133033	0.006060	0.291758	0.068872	0.781295	0.020046	0.041884	0.356907	0.031800



Supplemental Figure 1. Mean temperature and precipitation in Łódź city during experiment (April-September 2019)



Supplemental Figure 2. Fresh weight (FW; A) and dry weight (DW; B) partitioning calculated as shoot:root (S:R) ratio of the studied species of *Centaurea* grown in Podzol (p), Rendzina (r) or Rendzina with addition of 5 (r5) or 25 µmol Fe-HBED kg^{-1} soil (r25). Values (mean ± SD) with different letters for each parameter are significantly different between the treatments at $p < 0.05$ (n = 4; ANOVA with Tukey's HSD post-hoc test).

Praca P–4

W ostatnim etapie prac skupiono się na poznaniu wpływu chemicznych czynników edaficznych kontrastujących oba typy siedlisk (odczyn oraz dostępność żelaza, manganu i glinu) na zdolność nasion badanych gatunkach do zakończenia kiełkowania. Do doświadczeń wybrano 20 gatunków występujących w obrębie badanych typów muraw: *A. montanum*, *A. amellus*, *B. officinalis*, *C. scabiosa*, *C. stoebe*, *D. carthusianorum*, *D. deltoides*, *E. vulgare*, *G. cruciata*, *H. pilosella*, *H. perforatum*, *H. radicata*, *P. media*, *P. recta*, *P. grandiflora*, *R. acetosella*, *S. germanica*, *T. serpyllum*, *V. thapsus* i *V. teucrium*.

Badanie zaprojektowano jako serię niezależnych doświadczeń *in vitro* w warunkach kontrolowanych w oparciu o metodykę powszechną dla tego typu prac doświadczalnych (Baskin i Baskin, 2014). Pierwsze doświadczenie miało na celu określenie wymagań badanych gatunków względem odczynu (w warunkach buforowanych). Doświadczenie drugie i trzecie miało na celu zbadanie, odpowiednio, wpływu żelaza i manganu, podanego w formie jonowej (sól chlorkowa) oraz skompleksowanej (w chelacie HBED). Czwarte doświadczenie miało na celu określenie wpływu glinu w formie jonowej (sól chlorkowa). Zakres stężeń badanych czynników dobrano w oparciu o dane literaturowe, uwzględniając również zawartość badanych pierwiastków w roztworach glebowych zasadowych i kwaśnych gleb mineralnych, zbliżonych do gleb typowych dla danych typów muraw (Asher i Edwards, 1983; Marschner, 1988; Abedi i wsp., 2013; Bothe, 2015; Shao i wsp., 2017).

Niniejszy etap badań miał na celu przetestowanie hipotez badawczych związanych z jednorodnością reakcji na zadawane czynniki w obrębie danej grupy ekologicznej. Badanie miało posłużyć do testowania następujących hipotez – hipoteza pierwsza: wszystkie badane czynniki chemiczne wywierają wpływ na zdolność nasion do zakończenia kiełkowania (w przynajmniej jednej serii porównań różnica pomiędzy wariantem kontrolnym, a wariantem poddanym działaniu czynnika będzie istotna; H1); hipoteza druga: zarówno gatunki z piaszczystych muraw bezwapiennych, jak i kserotermicznych muraw wapieniolubnych posiadają specyficzne i powszechne dla grupy strategie kiełkowania, zgodne z warunkami ich siedlisk (nasiona wszystkich gatunków z piaszczystych muraw bezwapiennych będą kiełkowały istotnie lepiej w roztworze o niskiej wartości pH i roztworach zawierających więcej żelaza, manganu i glinu lub pierwiastek w formie jonowej, a nasiona wszystkich gatunków z kserotermicznych muraw wapieniolubnych będą kiełkowały istotnie lepiej w roztworze o wysokiej wartości pH i roztworach zawierających mniej żelaza, manganu i glinu lub pierwiastek w formie schelatowanej; H2); hipoteza trzecia: zdolność nasion do zakończenia kiełkowania oraz szybkość kiełkowania nie będzie skorelowana ze średnią masą diaspor (w obrębie grupy lub niezależnie od grupy; H3).

Seria doświadczeń wskazała jasno, że wszystkie badane czynniki wywierają istotny wpływ na zdolność nasion do wykiełkowania, a efekty mają charakter specyficzny gatunkowo. Oznacza to, że nie ma przesłanek o odrzucenia hipotezy pierwszej (H1). Rozważając amplitudę zmian powodowanych przez działanie badanych czynników, należy zaznaczyć, że odczyn oraz dostępność jonów glinu miały największy wpływ na proces kiełkowania, a dostępność i specjacja żelaza oraz manganu odgrywają drugorzędną rolę. Sugeruje to, że odczyn gleby i dostępność glinu, a nie dostępność żelaza i manganu są

głównymi filtrami odpowiedzialnymi za regulację procesu rekrutacji siewek. Na podstawie poprzednich etapów badań oraz danych literaturowych można również zaproponować, że dopiero w fazie juwenilnej dochodzi do selekcji osobników przez wszystkie cztery czynniki.

Siedliskowa próba interpretacji uzyskanych wyników odsłania kompleksowość czynników regulujących proces kiełkowania. Dlatego też, z rozlicznych przyczyn, hipoteza druga o specyfice siedliskowej strategii kiełkowania nie znajduje odzwierciedlenia w danych, co skłania do przyjęcia alternatywnej hipotezy mówiącej o gatunkowej specyfice reakcji na badane czynniki. Nasiona większości badanych gatunków (z obu badanych grup) są w stanie wykiełkować przy odczynie zdecydowanie odbiegającym od odczynu gleb na których najczęściej gatunki te bytują. Ponadto, nasiona części gatunków kalcyfilnych nie były w stanie wykiełkować przy wysokim odczynie, co sugeruje, że warunki umożliwiające wschód i wzrost wegetacyjny są różne (mniej prawdopodobne wyjaśnienie) lub gatunki te są wrażliwe na toksyczność powodowaną jonami sodu (bardziej prawdopodobne wyjaśnienie). Co więcej, nasiona tychże gatunków są w stanie wykiełkować w środowisku kwaśnym, ale warto zaznaczyć (w kontekście pierwszego etapu badań), że ich późniejszy wzrost jest mocno ograniczony. Takie same wyjaśnienie można zaproponować dla badanych kalcyfobów, których kiełkowanie w przeciwstawnych warunkach jest możliwe, ale dalszy wzrost jest znacząco ograniczony. Zarówno kalcyfile, jak i kalcyfoby reagują na zmieniającą się dostępność żelaza i manganu, jednakże zaobserwowane zmiany nie zachodzą zgodnie z linią podziału preferencji siedliskowych, tj. badane gatunki przejawiały swoistą dla siebie reakcję na dostępność i specjację obu badanych pierwiastków. Oznacza to, że tolerancja względem badanych metali na etapie kiełkowania nie może być szacowana wprost z wymagań tychże gatunków co do odczynu podłoża i *vice versa*. Podkreśla to zaobserwowaną w poprzednich etapach prac tendencję do rozdzielności wymagań względem odczynu i dostępności pierwiastków. Podobne wnioski można wysnuć analizując wyniki odnoszące się do wpływu glinu na proces kiełkowania. Stwierdzono, że dla większości gatunków stężenia glinu do 0,1 mmol · dm^{-3} nie mają silnego wpływu na zdolność nasion do zakończenia procesu kiełkowania. Oznacza to, że dostępność glinu w glebach umiarkowanie kwaśnych (pH ok. 5,0) nie może być czynnikiem silnie limitującym wschody kalcyfilów, ale jest to już bardzo prawdopodobne dla gleb kwaśnych, typowych dla piaszczystych muraw bezwapiennych, gdzie dostępność glinu zdecydowanie przekracza 1 mmol · dm^{-3} (Abedi i wsp., 2013).

Wyniki jasno wskazały, że masa diaspor nie przekłada się na zdolność nasion do wykiełkowania w badanych warunkach. Co prawda obserwowano przeciętną do wysokiej istotną korelację pomiędzy masą diaspor, a ostatecznym procentem kiełkowania i indeksem szybkości kiełkowania, ale wyniki odnosiły się wyłącznie do najniższych stężeń jonowej formy manganu (5 µmol · dm^{-3}) i glinu (0.01 mmol · dm^{-3}). Dlatego też, biorąc pod uwagę , że tylko 4 z 90 obliczonych współczynników korelacji było istotnych, istnieją poważne przesłanki do odrzucenia hipotezy trzeciej (H3) lub do opatrzenia jej zastrzeżeniem, że zależność między masą, a zdolnością do wykiełkowania ma miejsce wyłącznie przy niskim natężeniu badanych czynników.

W podsumowaniu niniejszego etapu badań przeprowadzono hierarchiczną analizę skupień, która wykazała, że żaden z czynników, który mógłby w sposób prawdopodobny tłumaczyć reakcję badanych gatunków nie jest w stanie wyjaśnić odpowiednio obserwowanych wzorców reakcji. Dlatego też użyteczność liczb wskaźnikowych Ellenberga, uogólnionej preferencji względem typu siedliska czy masy diaspor wydaje się mocno ograniczona w kontekście segregacji strategii kiełkowania. Oznacza to, że poznanie tej części niszy badanego gatunku, która odnosi się do zdolności do wykiełkowania w „miejscu bezpiecznym" (Harper, 1961), jest nieredukowalne do badania wyłącznie łatwo mierzalnych tzw. cech funkcjonalnych nasion (w tym masy).

Wyniki niniejszego etapu badań dowodzą istnienia w nasionach złożonych, immanentnych, lecz gatunkowo-specyficznych mechanizmów odpowiedzialnych za zdolność do radzenia sobie ze stresorami zewnętrznymi. Co prawda na obecnym etapie badań nie jest jasne które elementy strukturalno-funkcjonalne regulują zdolność nasion badanych gatunków do wykiełkowania w obliczu stresu, ale wydają się to być cechy inne niż ich podstawowe parametry wielkościowo-masowe (Larson i Funk, 2016). W kontekście badanych typów muraw dane te mają znaczenie zarówno poznawcze, jak i praktyczne. Z punktu widzenia badań podstawowych, biotop badanych muraw jest wielowarstwowym filtrem selekcyjnym, którego warstwy, choć działają symultanicznie, nie są równocenne (Jiménez-Alfaro i wsp., 2016). W tym kontekście, mikromozaiki „miejsc bezpiecznych" mogą wyraźnie wpływać na czasoprzestrzenną zmienność muraw. W kontekście praktyki, uzyskane dane mogą posłużyć do opracowania lepszych planów ochronnych dla obu typów siedlisk oraz wspomóc proces podejmowania decyzji w pracach z zakresu inżynierii środowiska.

W niniejszej części pracy doktorskiej zastosowano jedną metodę doświadczalną (ściśle kontrolowanie doświadczenie laboratoryjne) oraz, pomocniczo, cztery techniki analityczne (pH-metrię, spektrofotometrię, FT-IR oraz LC-MS/MS). W oparciu o uzyskane wyniki opublikowano autorską pracę w czasopiśmie *PeerJ* zatytułowaną „*Acidity and availability of aluminum, iron and manganese as factors affecting germination in European acidic dry and alkaline xerothermic grasslands*".

P–4

"Acidity and availability of aluminum, iron and manganese as factors affecting germination in European acidic dry and alkaline xerothermic grasslands"

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Acidity and availability of aluminum, iron and manganese as factors affecting germination in European acidic dry and alkaline xerothermic grasslands

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ABSTRACT

Germination ecology of 10 species from acidic dry grasslands and 10 species from alkaline xerothermic grasslands was studied. The seeds were subjected to different pH, iron (Fe), manganese (Mn) and aluminum (Al) treatments under controlled conditions. Effects of ionic (chlorides) and chelated forms (HBED chelates) of Fe and Mn were also compared. Final germination percentage (FGP) and index of germination velocity (IGV) were calculated. The results indicate that pH and extremely high availability of Al are the major edaphic filters regulating germination-based revegetation, while availability of Fe and Mn is of the secondary importance. Both chelates and ionic forms of Fe and Mn exerted similar effects on the ability of seeds to complete germination. It suggests that both chelates are not hazardous for early ontogenetic stages of plants. Neither group has group-specific adaptations pertaining to germination characteristics in the context of the studied chemical stimuli, which indicates a diversity of germination strategies and individual species-specific reactions to the tested factors.

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INTRODUCTION

Germination is a process which is controlled by environmental conditions (*Koornneef, Bentsink & Hilhorst, 2002*). Light, temperature and availability of water are known to be crucial factors affecting completion of germination of seeds (*Gibson, 2009*; *Graeber et al., 2012*). However, there are also many secondary (but important) abiotic factors affecting germination, such as availability and type of nitrogen-containing compounds and other macronutrients, acidity (pH), availability of micronutrients and nonessential trace/ballast elements as well as the amount and type of pollutants (*Graeber et al., 2012*; *Kranner & Colville, 2011*). Therefore, completion of germination that is observed in the field depends on a multifactorial interaction between environmental determinants. These stimuli directly affect metabolism of seeds and/or they are recognized and translated into a signal affecting

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hormonal equilibrium that prevents or triggers germination (*Nonogaki, 2017*; *Duermeyer et al., 2018*).

Grasslands are plant communities characterized with high solar irradiance and light heterogeneity (*Bakker, Blair & Knapp, 2003*). Light that reaches the soil surface of 'vegetation gaps', where completion of germination is most favored, is not strongly filtered due to the lack of dense canopy. Thus, it has an optimal spectrum that supports the germination process of photoblastic-positive plant species (*Deregibus et al., 1985*). Temperature of soil surface, where seeds of photoblastic-positive plant species complete germination, depends partially on physical-chemical properties of the soil (*Bothe, 2015*). Although non-calcareous sandy soils and calcareous soils can reach, respectively, 70 °C and 60 °C on a sunny day in summer (*Jentsch & Beyschlag, 2003*; *Bothe, 2015*), the temperatures of soil surface and diurnal amplitude of soil temperature in spring and autumn (when seeds of many herbs complete germination; *Czarnecka, 2004*) are lesser (*Burmeier et al., 2010*). This creates a safe environment in terms of major factors controlling the germination process. Soil solution, which makes imbibition of seeds possible, contains a wide spectrum of chemical compounds (*Marques et al., 1996*). Many of them interfere with the germination process, enhancing (*e.g.*, the appropriate form and dose of nitrogen; *Duermeyer et al., 2018*) or halting it (*e.g.*, due to toxicity of ions or due to supraoptimal osmoticum; *Kranner & Colville, 2011*).

Dry acidic grasslands from *Koelerio-Corynephoretea* Klika in Klika et V. Novák 1941 class and xerothermic alkaline grasslands from *Festuco-Brometea* Br.-Bl. et R.Tx. 1943 class are the priority types of habitats in Europe (*6120 and *6210, respectively; *Council Directive, 1992*). Plants from those habitats share two common traits, namely, tolerance to heat stress and high irradiance (*Leuchner & Ellenberg, 2017*). However, there are several iconic characteristics of each type of soil that differentiate between them (*Lee, 1999*; *Bothe, 2015*). Soils of dry acidic grasslands (mostly Podzols) are known to be remarkably acidic and to contain high concentrations of available Al, Mn and Fe due to low pH (*Abedi, Bartelheimer & Poschlod, 2013*; *Strawn, Bohn & O'Connor, 2020*). Depending on many soil-forming factors, concentration of Ca in non-calcareous sandy soils vary, but it is established that typical inland sandy soils (not affected by marine or alluvial deposition) are poor in this element (*Török et al., 2009*). Soils on which alkaline xerothermic grasslands develop (mostly Rendzinas) are completely opposite—they are rich in Ca and are characterized with low acidity due to composition of bedrock (in most cases bedrock is rich in $CaCO_3$; sometimes it originates from $CaSO_4$—then they are susceptible to acidification). Furthermore, due to high pH, several elements, *e.g.*, Fe, Mn and Al are precipitated in insoluble forms (*Bothe, 2015*; *Strawn, Bohn & O'Connor, 2020*). Considering two major characteristics of alkaline and acidic soils, namely availability of Ca and soil pH, plants on calcareous soils are recognized as calcicoles (sometimes referred to as basophiles or calciphiles), whereas those on silicate soils are calcifuges (or calciphobes/acidophiles, considering their reaction to Ca and pH; *Bothe, 2015*). Interestingly, the abovementioned factors (pH and availability of Ca, Fe, Mn and Al) were only rarely studied in the past as the factors affecting germination process in those types of grasslands.

It is known that different forms of mineral nutrients and ballast elements are not equally available for plants. For example, availability of Al is driven by soil pH, as soil solutions of acidic soils are remarkably rich in this element, while in alkaline soils its availability is definitely lesser (*Abedi, Bartelheimer & Poschlod, 2013*; *Bothe, 2015*). In the case of Fe and Mn, the most common species available for plants are their free ions (strictly limited source) and complexed forms (*Bothe, 2015*; *Shao et al., 2017*). Relatively much is known of Fe and Mn nutrition and effects of availability of these two elements on vegetative growth of higher plants (*Andresen, Peiter & Küpper, 2018*). Chelated Fe can be readily acquired and utilized (Strategy II plants) or reduced and then acquired by plants (Strategy I plants; *Grillet & Schmidt, 2017*). Furthermore, Fe and Mn are subjected to constant ligand (chelator) exchange (including competition of these metals for a given chelator), which probably shifts their availability in soil environment (*Yehuda et al., 1996*; *Duckworth, Bargar & Sposito, 2009*). The phenomenon of metal chelation (by biological as well as synthetic chelators, *e.g.*, *N*,*N*'-di(2-hydroxybenzyl)ethylenediamine-*N*,*N*'-diacetic acid; HBED) underlies research on soil chemistry and nutrition of plants (where chelates can be used as a tool; *Strawn, Bohn & O'Connor, 2020*; *Wala et al., 2020*), but it was also found to be a remedy for losses of productivity in agriculture (*Venturas et al., 2014*). Even apart from the scientific and economical significance of this problem, there are some other practical issues associated with the influence of ionic and chelated forms of Fe and Mn on plants. Although the usage of metal chelates in agriculture and horticulture has gained popularity in recent years, not much is known about their environmental effects and safety, including their influence on the early ontogenetic stages of plants. According to the available data, HBED chelates were not studied in this context. Furthermore, Mn-HBED was never investigated in the context of plant physiology, thus nothing is known about its mode of action and safety.

Although functional ecology of seeds, including effects of habitat sieves (*e.g.*, edaphic factors) has recently gained more attention in plant ecology (*Poschlod, 2020*), there is only fragmental information about germination patterns that can be observed in temperate grasslands. For example, germination-related requirements pertaining to pH of calcicole and calcifuge plant species from temperate grasslands are hardly known. Furthermore, although influence of metals on the completion of germination was solicitously studied and reviewed, information pertaining to the role of Al as well as Fe and Mn (both in ionic and complexed forms) in this process is very scarce (*Kranner & Colville, 2011*). So far, some studies addressed only the problem of influence of Al toxicity on the ability of seeds to complete germination in some wild-living plant species (*Abedi, Bartelheimer & Poschlod, 2013*). Therefore, we performed the experiments to shed light on less studied aspects of germination ecology of the selected plant species from contrasting European grasslands (Table 1). The following hypotheses were addressed: (1) calcicole plant species (in terms of their centers of abundance) prefer alkaline pH while calcifuge plant species prefer acidic pH for completion of germination; (2) availability of iron (Fe-HBED and $FeCl_3$) and manganese (Mn-HBED and $MnCl_2$) impacts germination-related characteristics, and (3) increasing concentration of $AlCl_3$ affects germination, whereby acidophilous species are more resistant than basophilous ones. The following questions were addressed: (1) do pH

Table 1 List of the studied plant species, their growth forms, seed characteristics, habitat preferences and ecological indicator values describing their realized centers of abundance.

Species	Abbreviation	Family	Growth Form[a]	Seed size (mm)[b]	Seed weight (mg)[c]	Preference to soil pH[d]	Ellenberg's indicator value[e]					
							L	T	K	F	R	N
Alyssum montanum L.	Amo	Brassicaceae	P	1.5–1.9 × 1.1–1.3	0.369 ± 0.002	A	9	6	4	2	7	1
Aster amellus L.	Aam	Asteraceae	P	3.4–3.8 × 1.5–1.8	1.010 ± 0.029	B	8	6	6	4	9	3
Betonica officinalis L.	Bof	Lamiaceae	P	2.7–3.1 × 1.3–1.5	1.147 ± 0.035	B	7	6	5	*	0	3
Centaurea scabiosa L.	Csc	Asteraceae	P	4.5–5.0 × 2.0–2.2	4.815 ± 0.211	B	7	0	3	3	8	4
Centaurea stoebe Tausch	Cst	Asteraceae	B	2.5–3.0 × 1.2–1.4	2.146 ± 0.033	A	8	7	5	2	8	3
Dianthus carthusianorum L.	Dca	Caryophyllaceae	P	2.0–2.4 × 1.5–1.8	0.283 ± 0.008	A	8	5	4	3	7	2
Dianthus deltoides L.	Dde	Caryophyllaceae	P	1.1–1.6 × 0.7–1.0	0.106 ± 0.010	A	8	5	4	3	3	2
Echium vulgare L.	Evu	Boraginaceae	B/P	2.4–2.8 × 1.5–1.8	2.242 ± 0.048	A	9	6	3	4	8	4
Gentiana cruciata L.	Gcr	Gentianaceae	P	1.1–1.3 × 0.5–0.6	0.090 ± 0.003	B	7	6	4	3	8	3
Hieracium pilosella L.	Hpi	Asteraceae	P	2.1–2.3 × 0.5–0.5	0.147 ± 0.002	A	7	0	3	4	0	2
Hypericum perforatum L.	Hpe	Hypericaceae	P	1.0–1.2 × 0.5–0.6	0.076 ± 0.002	A	7	6	5	4	6	3
Hypochaeris radicata L.	Hra	Asteraceae	P	5.0–10.0 × 0.5–0.6	0.962 ± 0.025	A	8	5	3	5	4	3
Plantago media L.	Pme	Plantaginaceae	P	1.7–2.1 × 1.0–1.2	0.249 ± 0.007	B	7	0	7	4	7	3
Potentilla recta L.	Pre	Rosaceae	P	1.3–1.7 × 1.9–2.1	0.385 ± 0.018	B	9	7	5	3	5	2
Prunella grandiflora (L.) Scholler	Pgr	Lamiaceae	P	1.7–1.9 × 1.5-1.7	1.217 ± 0.031	B	7	0	5	3	8	3
Rumex acetosella L.	Rac	Polygonaceae	P	1.4–1.8 × 1.1–1.3	0.200 ± 0.013	A	8	5	3	4	2	2
Stachys germanica L.	Sge	Lamiaceae	B	1.9–2.2 × 1.4–1.6	1.392 ± 0.065	B	7	7	4	3	8	5
Thymus serpyllum L.	Tse	Lamiaceae	S	0.6–0.8 × 0.5–0.7	0.143 ± 0.004	A	7	6	5	2	5	1
Verbascum thapsus L.	Vth	Scrophulariaceae	B	0.8–1.0 × 0.5–0.6	0.131 ± 0.004	A	8	0	3	4	7	7
Veronica teucrium L.	Vte	Plantaginaceae	P	1.3–1.6 × 1.1–1.2	0.320 ± 0.022	B	7	6	5	3	8	2

Notes.

Nomenclature of studied species follows the Plant List (http://www.theplantlist.org).

[a] growth form of a given species, where: B –biennial herb, P –perennial herb, S –subshrub/semishrub.

[b] seed size follows the literature (*Bojňanský & Fargašová, 2007*).

[c] seed weight was measured in this study (determined by weighing 100 air-dried seeds; mean ± SD, $n = 4$).

[d] soil preference of the studied species estimated prior the experimental phase (basing on criteria presented in the article), where: A—acidophilous species from dry acidic grasslands, B –basophilic species from xerothermic alkaline grasslands.

[e] ordinal scale (1–9) of Ellenberg's Indicator Values (*Ellenberg, 1991*), where: L—light requirements ranging from 7 to 9, where 7 indicates well-lit/slightly-shaded conditions (c.a. 30% of relative illumination) and 9 indicates full light conditions (>50% of relative illumination); T—temperature requirements ranging from 5 to 7, where 5 where 5 indicates species preferring moderately cool to warm conditions (characteristic of montane and submontane conditions, mostly southern Fennoscandia) and 7 indicates species preferring warm conditions (characteristic of North European Plain); K—continentiality requirements ranging 2 to 5, where 2 indicates atlantic conditions and 5 indicates subatlantic to subcontinental conditions; F—soil moisture requirements ranging from 2 to 5, where 2 indicates dry soils and 5 indicates moist soils; R—soil pH requirements ranging from 2 to 9, where 2 indicates extremely acidic to acidic soils and 9 indicates extremely alkaline soils originating from limestones; N—soil nutrient requirements ranging from 1 to 7, where 1 indicates extremely infertile soils and 7 indicates fertile soils; 0—indifferent behaviour, wide amplitude or unequal behaviour in different areas; *-uncertain and not fully described behaviour.

and availability of Fe, Mn and Al influence the ability of seeds to complete germination? (2) do HBED-chelated Fe and Mn affect germination to a greater extent than their chlorides? (3) is Al a real threat to germination-based revegetation?

MATERIALS & METHODS

Criteria of species selection and collection of seeds

The plant species used in this study were selected based on their habitat preferences (Table 1). In total, 20 species were selected (10 from acidic dry grasslands and 10 from alkaline xerothermic grasslands). Their occurrence on the respective sites in central Europe was also taken into consideration (*Czyzewska, 1992*; *Mucina & Kolbek, 1993*; *Matuszkiewicz, 2001*; *Kącki, Czarniecka & Swacha, 2013*; *Hegi, 1931*). According to the available literature (*Ellenberg, 1991*), all the selected species are light-loving plants that can be found in warm and relatively dry sites characterized with low nitrogen soil status (Table 1). Furthermore, the selected plant species belong to different plant families. With such a wide selection of species, we intended to examine the most common, as well as the most valuable, plants that can be found in the studied communities.

The propagules (fruits—achenes for the studied species from Asteraceae and Rosaceae, nutlets for the studied species from Boraginaceae and Lamiaceae –and seeds for rest of the studied species; therein after referred to as seeds) were manually collected from plants cultivated in the Didactic-Experimental Garden of Faculty of Biology and Environmental Protection, University of Lodz (51°78′N, 19°48′E; 223 m a.s.l.), at full maturity stage in summer, 2019 (exact timing depended on the species). The seeds were gathered from at least 20 healthy and representative plant individuals growing in homogenous and optimal conditions (matching individual requirements for growth and reproduction of each species). The seeds were stored in paper bags in the laboratory for 14 days (at a relative humidity of 30%). Then they were inspected (all malformed, discolored and damaged seeds were removed) and weighed (four lots of 100 randomly selected seeds). Dimensions of the seeds were taken from the literature (*Bojñanský & Fargašová, 2007*). Then, the seeds were stratified in a refrigerator (5 °C) for 16 weeks.

General germination procedures

The ability of seeds to complete germination was tested on glass Petri dishes ($\phi = 5$ cm). The dishes were lined with two pieces of filter paper (Whatman no. 1; GE Healthcare, Chicago, IL, United States). The seeds were mixed before the tests in order to fulfill the randomization requirement. On each dish, 25 seeds were placed and moistened with 1.5 cm^3 of the tested solutions. Subsequently, the dishes were sealed with parafilm to maintain homogenous moisture and humidity and placed in a germination cabinet. All experiments were conducted under identical conditions of fully-controlled thermo-photoperiod (16 h of light phase at 25 °C and 8 h of dark phase at 15 °C; light was supplied with white fluorescent lamps, PAR = 40 μmol m^{-2} s^{-1}, measured with FluorPen; PSI, Drasov, Czech Republic). Those photo-thermal conditions were selected due to the fact that many of the species we studied complete germination in spring (*Czarnecka, 2004*), when air temperature is relatively high in central Europe. This thermoperiod was previously used

in other multispecies investigations focusing on a similar topic (*Tudela-Isanta et al., 2018*) and it is within optimal temperature range allowing completion of germination of many species from the temperate zone, including those from temperate grasslands (*Baskin & Baskin, 1988*; *Dürr et al., 2015*; *Ladouceur et al., 2019*). Furthermore, based on the data pertaining to the seed weight of the species we studied (in most cases the seeds weighed <1.5 mg; Table 1), they are likely to be photoblastic positive or at least photoblastic neutral (*Jankowska-Blaszczuk & Daws, 2007*), which was confirmed by preliminary tests showing that all the species we studied were able to complete germination under the described conditions. All tested solutions were prepared using distilled water (conductivity <0.08 μS cm^{-1}). The pH values of each solution (Table S1) were measured with digital pH-meter (CP-401; Elmetron, Zabrze, Poland). Completion of germination was counted daily for 21 d. During the experiments, the seeds were monitored for any signs of their unviability (seed softness and brownish embryo color), however no such situation was observed.

Experiment 1: Effects of pH

Phosphate buffer (KH_2PO_4:Na_2HPO_4; 0.066 mol dm^{-3}) was used in this study as a medium ensuring the desired pH. It was the buffer of choice due to its relatively wide range of available pH values. Moreover, it is not toxic to cells and its acidity does not change substantially with change of temperature. Furthermore, each used phosphate species co-occurs in soil with a given soil pH, *i.e.*, $H_2PO_4^-$ occurs predominantly in acidic soils, while HPO_4^{2-} in alkaline ones (*Strawn, Bohn & O'Connor, 2020*), thus it reflects the available forms of P in the studied grasslands (*Leuchner & Ellenberg, 2017*). The effect of pH on germination was studied within the pH range of 5.0–8.0 in increments of 1.0 pH unit (similar to a setup used elsewhere; *Tudela-Isanta et al., 2018*). The buffers were prepared using analytical grade salts. The experiment was run according to the setup described in 'General Germination Procedures'.

Experiment 2: Effects of ionic and complexed iron

Fe-HBED chelate (*N*,*N*'-di(2-hydroxybenzyl)ethylenediamine-*N*,*N*'-diacetic acid iron(III) sodium salt; 7% Fe; synthesized according to European Patent Application EP20461587.6 by PPC ADOB, Poland, and authenticated using LC-MS/MS, FTIR and UV-VIS analyses; Fig. S1) was used as a complexed form of Fe (chelation rate 1:1 with favored Fe(III) oxidation state). This agent is characterized by high stability under a wide range of conditions, including pH, thus it can be used as a tool simulating natural metal chelation under high soil pH. Anhydrous $FeCl_3$ of analytical quality was used as the ionic form of Fe^{3+}. The effect of Fe was evaluated using isomolar concentrations of both compounds (0, 5 and 25 μmol dm^{-3}). This range of concentrations was the same as that used in our previous studies evaluating physiological response of plants at vegetative stage (*Wala et al., 2020*). These concentrations were also within the range maintaining optimal plant growth under hydroponic conditions (*Venturas et al., 2014*; *Kasozi et al., 2019*) and are higher than the concentration of available Fe in a soil solution of Rendzinas (picomolar/nanomolar values; *Bothe, 2015*). The experiment was run according to the setup described in 'General Germination Procedures'.

Experiment 3: Effects of ionic and complexed manganese

The complexed form of Mn (chelation rate 1:1 with favored Mn(III) but possible Mn(II) oxidation state; *Pinto et al., 2019*) was supplied as Mn-HBED (*N,N*'-di(2-hydroxybenzyl)ethylenediamine-*N,N*'-diacetic acid manganese (II) sodium salt; 7% Mn; synthesized by PPC ADOB, Poland). HBED was selected as Mn-complexing agent in order to reduce bias resulting from comparison of different chelators (in this case Fe-HBED vs Mn-HBED). Furthermore, high stability of HBED under alkaline conditions simulates chelation process in the studied type of community (alkaline xerothermic grassland).

Mn-HBED was synthesized as following: a 250 cm^3 flask with a magnetic stirrer, a pH-meter and a reflux condenser was charged with 100 g of water and 23.96 g (0.05 mol) of *N,N*'-di(2-hydroxybenzyl)ethylenediamine-*N,N*'-diacetic acid (HBED) monohydrochloride (purity 88.6%). Next, 1 mol dm^{-3} solution of sodium hydroxide (NaOH) was added to the slurry until pH 7 was reached. After complete homogenization, 10.39 g (0.0525 mol) of $MnCl_2 \cdot 4H_2O$ (purity 99%) in 30 ml of water was added in portions. During the addition of $MnCl_2 \cdot 4H_2O$, pH was maintained in range of 6–8 using 1 mol dm^{-3} solution of NaOH. After the complete addition of $MnCl_2 \cdot 4H_2O$, mixture was stirred for 1 h at room temperature. Then, the mixture was alkalized to pH 11 using a 1 mol dm^{-3} solution of NaOH. The solution was left without stirring for 24 h in darkness to allow excess Mn to precipitate as oxide then filtered through a cellulose filter and the solvent was removed *in vacuo*. Finally, 23.50 g of *N,N*'-di(2-hydroxybenzyl)ethylenediamine-*N,N*'-diacetic acid, manganese(II) sodium salt, were obtained. The product assay was 7.33% of manganese according to EN 16963:2018-03. Mn-HBED was authenticated using LC-MS/MS, FTIR and UV–VIS analyses (Fig. S1).

The ionic form of Mn was supplied with analytical grade $MnCl_2 \cdot 4H_2O$. The effect of Mn was evaluated using concentrations equal to those used in Experiment 2 (0, 5 and 25 μmol dm^{-3}). These concentrations were used in order to (1) compare the effects of Fe and Mn and (2) simulate Mn-over-supplemented environment, more Mn-rich than a soil solution (where average availability of Mn ranges c.a. 0.1–10 μmol dm^{-3} soil solution and in near-alkaline and acidic soils is close to 0.4 and 16.0 μmol dm^{-3} soil solution, respectively; *Marschner, 1988*; *Shao et al., 2017*) and artificial media optimal for soil-less plant cultivation (*e.g.*, Hoagland solution, where Mn concentration in full strength medium is equal to 9 μmol dm^{-3}; *Asher & Edwards, 1983*). The experiment was run according to the setup described in 'General Germination Procedures'.

Experiment 4: Effects of aluminum

The test was conducted according to the available data reporting a nonlinear relation between exchangeable Al and the pH of soil (*Abedi, Bartelheimer & Poschlod, 2013*). Analytical grade $AlCl_3 \cdot 6H_2O$ was used as source of ionic Al (*Abedi, Bartelheimer & Poschlod, 2013*). The effect of Al on seed germination was evaluated using 0.00, 0.01, 0.10, 1.00 and 10.00 mmol dm^{-3} solutions, which reflects the availability gradient of this element from alkaline to acidic soils (*Abedi, Bartelheimer & Poschlod, 2013*). Tested solutions were prepared using the serial dilution method. The experiment was run according to the setup described in 'General Germination Procedures'.

Calculation of germination-related parameters

To determine reactions of the species we studied, the final germination percentage (FGP; ranging 0–100%) and index of germination velocity (IGV, known as modified Timson's index, ranging 0–100; *Khan & Ungar, 1997*) were calculated. The greater values of FGP and IGV, the greater ability to complete germination or the more rapid germination, respectively. The IGV was calculated with the germinationmetrics package v. 0.1.3 (*Aravind et al., 2020*) run in R software (v. 3.5.2, 64 bit version; *R Core Team, 2018*) using preprogrammed equations of the package.

Statistical analysis

All experiments were replicated four times ($n = 4$). Normality of the data was analyzed with the Kolmogorov–Smirnov's test and homogeneity of variances was analyzed with the Brown–Forsythe's test. To detect differences among treatments within a given species, one-way ANOVA followed by the Bonferroni post-hoc test was mounted (differences were accepted as statistically significant at $p < 0.05$). To inspect effects of the studied factors, two-way ANOVA (Experiment 2 and Experiment 3) and three-way ANOVA (comparison between Experiment 2 and Experiment 3) were used. Differences between effects of isomolar solutions of Fe and Mn (Experiment 2 and Experiment 3, respectively) were detected with two-way ANOVA followed by the Bonferroni post-hoc test (differences were accepted as statistically significant at $p < 0.05$). Correlation between seed weight and germination reaction was calculated with Spearman's sum rank test. Analysis was conducted for all tested species ($n = 20$) as well as for separated groups of acidophilous ($n = 10$) and basophilous ($n = 10$) species. For each FGP value, the data were normalized to FGP values recorded at pH = 7 (Experiment 1) or control (null concentration) conditions (Experiments 2–4). Correlation was considered as statistically significant at $p < 0.05$. All statistical analyses were performed with Statistica^{TM} v. 13.3 (Tibco Software Inc.; Palo Alto, CA, USA).

To find out if there are similarities between the studied species (segregation of species on the basis of germination strategies), FGP-based hierarchical cluster analysis (HCA) was performed, merging data from Experiments 1–4. For each species, in order to reduce bias from the species-specific reaction (namely due to differences in completion of germination under control conditions), the data were normalized by calculation of ratio of FGP value at given treatment and FGP value recorded at pH = 7 (Experiment 1; due to neutral acidity and the most proportional KH_2PO_4: Na_2HPO_4 ratio) or control (null concentration) conditions (Experiments 2–4) as it was done for calculation of correlations. Phenograms were constructed using Ward's method of row clustering and Manhattan distance and the tightest clusters were presented first. HCA analysis was performed using ClustVis online tool (*Metsalu & Vilo, 2015*) with mean values from normalization, as proposed recently (*Chen et al., 2020*).

RESULTS

Experiment 1: Effects of pH

FGP values did not depend on pH of the buffered solutions in Cst and Dde (Fig. 1A), whilst in the other studied species they were differentially regulated. Acidophilous species showed preference to acidic conditions (Amo, Hpi, Hpe, Hra, Tse and Vth) and neutral conditions (Evu) or the preference was not clear (Rac; Fig. 1A). On the other hand, basophiles showed 4 general types of reactions: (1) preference for near-neutral pH (Csc and Gcr), (2) good completion of germination at acidic and neutral pH with strongly marked inhibition of completion of germination at alkaline pH (Aam, Bof, Pme, Pre, Pgr and Vte), (3) relatively wide tolerance of the tested pH values of the buffered solutions with marked acidophilism (Dca) and (4) strong preference to neutral and alkaline conditions with marked decrease in FGP under acidic conditions (Sge) (Fig. 1A). Interestingly, Tse showed marked decrease in FGP at optimal pH value, when compared to null concentrations in Experiments 2–4 (Fig. 1).

IGV values were nearly proportional to corresponding FGP values with two exceptions. In the case of Aam and Gcr completion of germination was slow (Fig. 1B). Germination speed in Gcr did not dependent on the buffer composition, as this species completed germination with similar velocity in Experiments 2–4, whilst in Aam this was probably caused by the compounds present in the buffered solutions (KH_2PO_4 and Na_2HPO_4) or their concentration (when compared to IGV values from Experiments 2–4).

Experiment 2: Effects of ionic and complexed iron

The tested Fe-containing compounds affected completion of germination of the studied species except that of Bof, Csc, Cst and Tse (Fig. 2A). Considering the $FeCl_3$-treated seeds, reduction of completion of germination was observed both after the treatment with 5 μmol dm^{-3} (Gcr, Hpi and Pgr) and with 25 μmol dm^{-3} (Aam, Dca, Dde, Gcr, Hpi, Hra, Pgr, Sge, Vth and Vte; Fig. 2A). Similar results were observed for Fe-HBED-treated seeds, as the solution of 5 μmol dm^{-3} negatively affected completion of germination in Dca, Dde, Evu, Hpi and Hra, whilst that of 25 μmol dm^{-3} reduced completion of germination in Dca, Dde, Evu, Hpi, Hpe, Hra, Pme, Pre, Sge and Vth (Fig. 2A). Both $FeCl_3$ (5 and 25 μmol dm^{-3}) and Fe-HBED (5 μmol dm^{-3}) positively affected completion of germination in Amo (Fig. 2A).

Effects of the tested conditions on FGP and IGV values were similar (Fig. 2B). With the exception of Gcr which germinated slowly but very successfully (Fig. 2A).

Two-way ANOVA showed that the type of Fe-bearing compound influenced FGP values in nine species (Amo, Aam, Evu, Gcr, Hpi, Hra, Pre, Pgr and Rac), whilst the dose of tested compound affected 10 species (Amo, Aam, Csc, Dca, Dde, Hpe, Hra, Sge, Vth and Vte; Table S2). Interaction between the type and the dose of the tested compounds affected significantly 4 species in total (Amo, Dde, Hpe and Hra; Table S2). IGV values were significantly affected by Fe type in 11 species (Amo, Aam, Cst, Evu, Gcr, Hpi, Hpe, Hra, Pre, Pgr and Rac) and by Fe dose in 12 species (Amo, Aam, Csc, Dca, Dde, Hpe, Hra, Pme, Rac, Sge, Vth and Vte; Table S2). Interaction between these factors affected IGV values in four species (Amo, Dde, Hpe and Hra; Table S2).

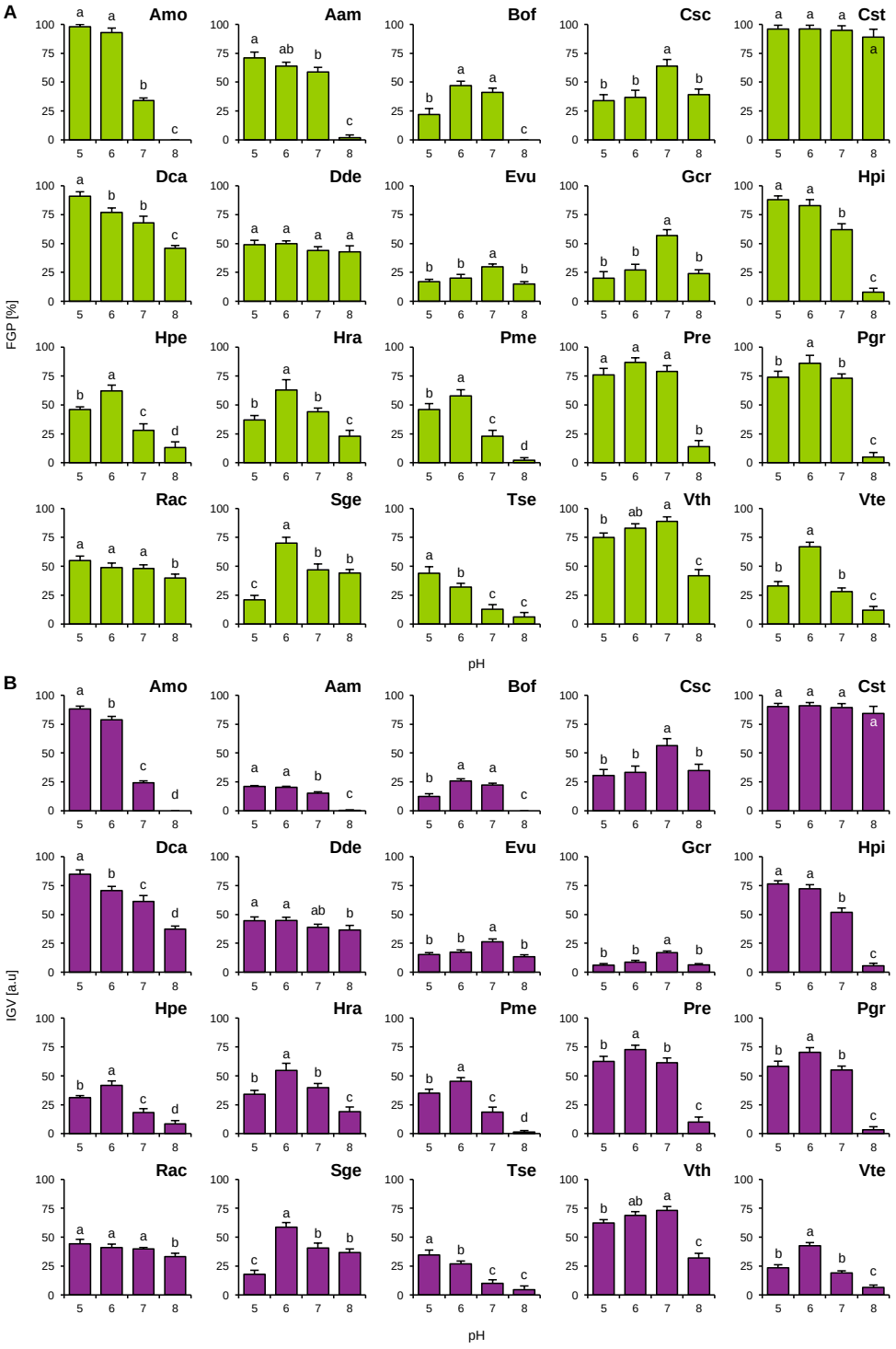

Figure 1 Effect of pH on the final germination percentage (FGP; A) and index of germination velocity (IGV; B) of the tested species from acidic dry and alkaline xerothermic grasslands. Values are the mean ± SD ($n = 4$). Different letters indicate significant differences between groups (ANOVA with Bonferroni post-hoc test, $p < 0.05$).

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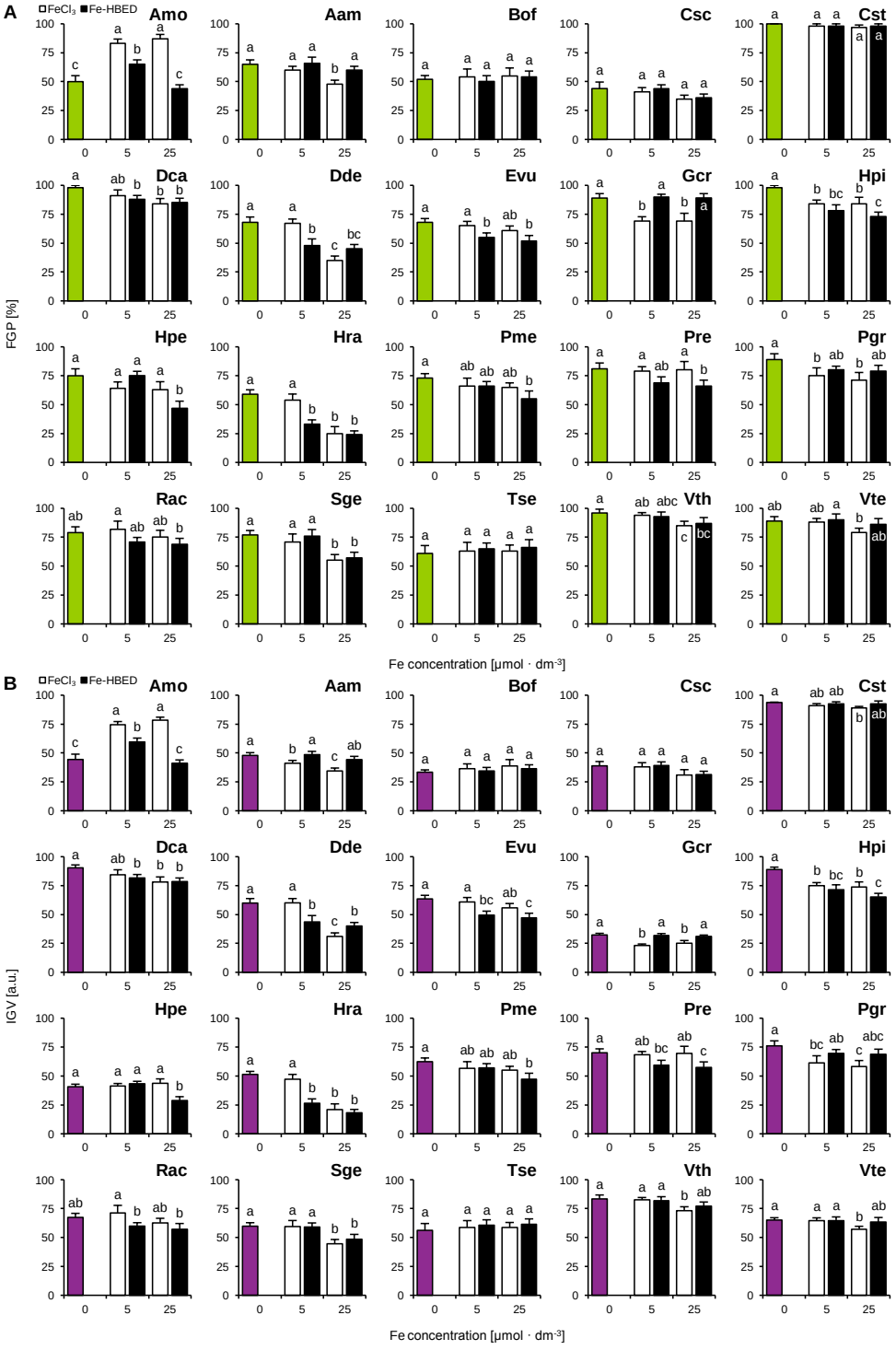

Figure 2 **Effect of ionic and chelated iron ($FeCl_3$ and Fe-HBED) on the final germination percentage (FGP; A) and the index of germination velocity (IGV; B) of the tested species from acidic dry and alkaline xerothermic grasslands.** Values are the mean ± SD ($n = 4$). Different letters indicate significant differences between groups (ANOVA with Bonferroni post-hoc test, $p < 0.05$).

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Experiment 3: Effects of ionic and complexed manganese

Mn treatments had no effects on FGP values in Cst, Dca, Gcr, Hpi, Pre, Tse, Vth and Vte (Fig. 3A). On the other hand, $MnCl_2$ solution of 5 μmol dm^{-3} had a negative effect on the ability of seeds to complete germination in Aam, Bof, Csc, Hpe and Pme (Fig. 3A). Decreases in FGP values were observed in Amo, Aam, Bof, Csc, Hpe, Hra, Pme, Rac and Sge due to application of 25 μmol dm^{-3} $MnCl_2$ solutions (Fig. 3A). Negative effects of Mn-HBED were also observed in the species treated with 5 μmol dm^{-3} (Aam, Csc, Dde, Hpe and Pme) as well as with 25 μmol dm^{-3} solutions (Aam, Csc, Dde, Evu, Hpe, Hra, Pme and Pgr; Fig. 3A).

The changes of IGV values were similar to those in FGP (Fig. 3B). Similar to what was shown in Experiment 2, Gcr proved to be an exceptionally slow germinator (Fig. 3B).

Two-way ANOVA showed that the type of Mn-containing agent affected five species (Aam, Bof, Dde, Pme and Rac) and Mn dose affected significantly four species (Amo, Csc, Hra and Rac; Table S2). Only one species was affected significantly by the interaction between the tested factors (Rac; Table S2). Similar effects were observed for IGV values (where Aam, Bof, Dde, Pme and Rac were affected by Mn type and Amo, Csc, Hra and Rac were affected by Mn dose; Table S2), but in the case of interaction between Mn type and dose, two species were affected significantly (Gcr and Rac; Table S2).

Comparison of iron and manganese as stimuli for germination

Five species (Cst, Pre, Sge, Tse and Vth) had similar tolerance to both metals (Table S3). Seven species completed germination better in Fe than in Mn solutions (Amo, Aam, Bof, Csc and Rac in the case of chlorides; Aam, Csc, Gcr and Hpe of HBED-chelated forms) while for the others quite the opposite was the case (*e.g.*, Dca for 25 μmol dm^{-3} of chloride; Dde, Hra and Vte for 25 μmol dm^{-3} of chlorides; Pgr for both doses of chlorides; Hpi and Rac for both concentrations of HBED-chelated forms; Table S3).

Three-way ANOVA showed that the element (Fe/Mn) affected FGP values in 12 species (Amo, Aam, Bof, Csc, Dca, Dde, Evu, Hpi, Hpe, Hra, Pgr and Vte), the type of metal bearing compounds influenced FGP values significantly in 11 species (Amo, Aam, Bof, Csc, Dde, Evu, Gcr, Hpi, Hra, Pme and Pre) and the dose affected FGP values in nine species (Amo, Aam, Csc, Dde, Hpe, Hra, Rac, Sge and Vth; Table S4). Interactions between two factors affected 5–11 species and interaction between all three factors affected only five species (Amo, Dde, Hpe, Hra and Rac; Table S4). Very similar effects were observed for IGV values (Table S4).

Experiment 4: Effects of aluminum

Six species (Amo, Cst, Dca, Rac, Tse and Vth) completed germination well in all tested concentrations of Al, as their FGP values did not differ significantly (Fig. 4A). The solutions ≥ 0.01 mmol dm^{-3} inhibited completion of germination in Pgr and Sge, ≥ 0.1 mmol dm^{-3} and greater solutions inhibited completion of germination in Aam, Dde, Hpi and Pme, ≥ one mmol dm^{-3} and greater solutions reduced FGP values in Bof, Hpe and Pre and 10 mmol dm^{-3} solutions reduced FGP values in Gcr, Hra and Vte (Fig. 4A). Interestingly, Al-stimulated completion of germination was observed in Hpe (0.01 mmol dm^{-3}) and in

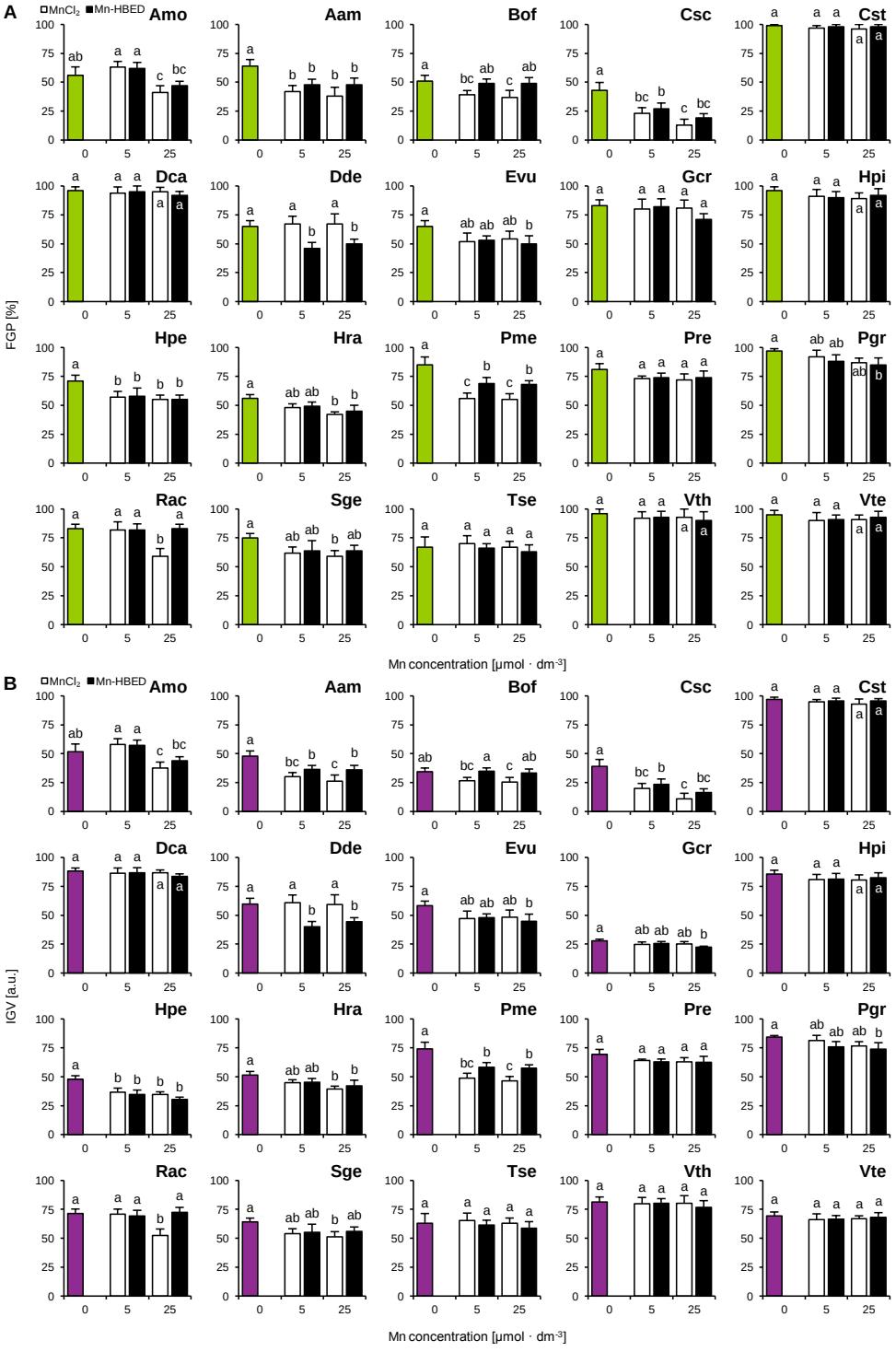

Figure 3 **Effect of ionic and chelated manganese ($MnCl_2$ and Mn-HBED) on the final germination percentage (FGP; A) and index of germination velocity (IGV; B) of the tested species from acidic dry and alkaline xerothermic grasslands.** Values are the mean ± SD ($n = 4$). Different letters indicate significant differences between groups (ANOVA with Bonferroni post-hoc test, $p < 0.05$).

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Csc (10 mmol dm^{-3}; Fig. 4A). The observed patterns of FGP responses were similar to those of IGV (Fig. 4B).

Classification of germination requirements and relations of seed traits to germination

Seed weight was negatively correlated with FGP values only when the seeds were subjected to 5 μmol dm^{-3} $MnCl_2$ ($R = -0.447$; $p = 0.048$; Table S5). IGV values were also negatively correlated with weight only when the seeds were subjected to 0.01 mmol dm^{-3} $AlCl_3$ ($R = -0.503$; $p = 0.024$ for all the studied species and $R = -0.648$; $p = 0.043$ for acidophiles; Table S5). All other correlations were insignificant ($p > 0.05$; Table S5).

Hierarchical cluster analysis (HCA) showed that acidity and extremely high concentration of Al (10.00 mmol dm^{-3}) were major factors allowing clustering of the studied species, whilst the other studied treatments had only minor contribution to species segregation (Fig. 5). According to the HCA, five major groups of species were distinguished: (1) species with preference for sub-neutral acidity (pH = 6) that are able to tolerate high doses of Al (Csc, Evu, Gcr and Vth); (2) species with marked inhibition of germination completion at pH = 8 and reduced ability to complete germination in the presence of Al (Sge, Bof, Pre and Pgr); (3) species with rather wide tolerance to acidity and not strongly affected by Al (Dde and Hra); (4) species with marked preference for acidic conditions and no obvious reaction to Al (Amo, Tse, Hpe, Pme and Vte); and (5) species that prefer acidic conditions (with marked susceptibility to alkalization) and no reaction to Al (Rac, Aam, Cst, Dca and Hpi; Fig. 5). The estimated soil preference and seed weight were not good predictors of completion of germination under the tested conditions, as clustering did not distinguished sharp and homogenous groups (Fig. 5). On the other hand, three first clusters were relatively well segregated considering R Ellenberg's Indicator Value (R EIVs), but the remaining two clusters did not show any logical pattern (Fig. 5).

DISCUSSION

It is commonly accepted that grasslands established on alkaline calcareous soils are composed of calcicole/calciphile/basophile plant species, while those established on acidic sandy soils are composed of calcifuges/calciphobes/acidophiles (*Bothe, 2015*). However, this concept was only occasionally tested to show its viability (*Bothe, 2015*), especially considering germination requirements. Although drastic changes of soil chemistry (*e.g.*, pH –(*Wagner et al., 2017*) –and availability of nutrients, most remarkably Fe, Mn and Al) in Rendzinas and Podzols are not very likely, slightly varying physical-chemical properties of soil (including its patchy structure; *Cain et al., 1999*) create a mosaic of microhabitats which contributes to plant diversity (*Leuchner & Ellenberg, 2017*). It suggests that some species within a given habitat type can be more/less calciphilic (or acidophilic) than others or show an undifferentiated reaction to soil pH and availability of the studied metals (in terms of germination requirements). The presented work is in line with this conception. It must be however noted that further validation of the presented findings on annual dicotyledonous plants as well as grasses is needed in order to fully describe the studied plant communities.

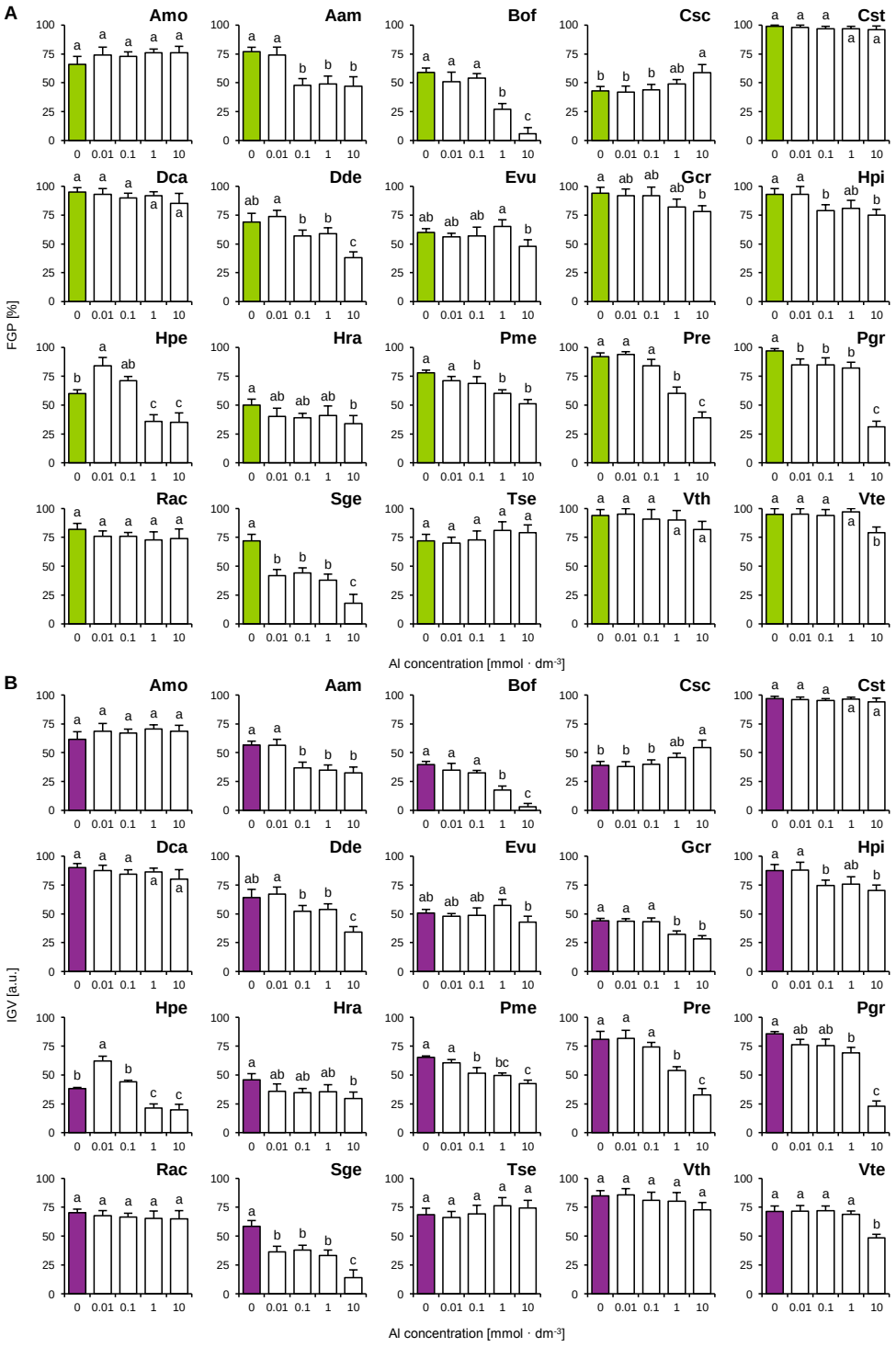

Figure 4 **Effect of aluminum ($AlCl_3$) on the final germination percentage (FGP; A) and index of germination velocity (IGV; B) of the tested species from acidic dry and alkaline xerothermic grasslands.** Values are the mean ± SD ($n = 4$). Different letters indicate significant differences between groups (ANOVA with Bonferroni post-hoc test, $p < 0.05$).

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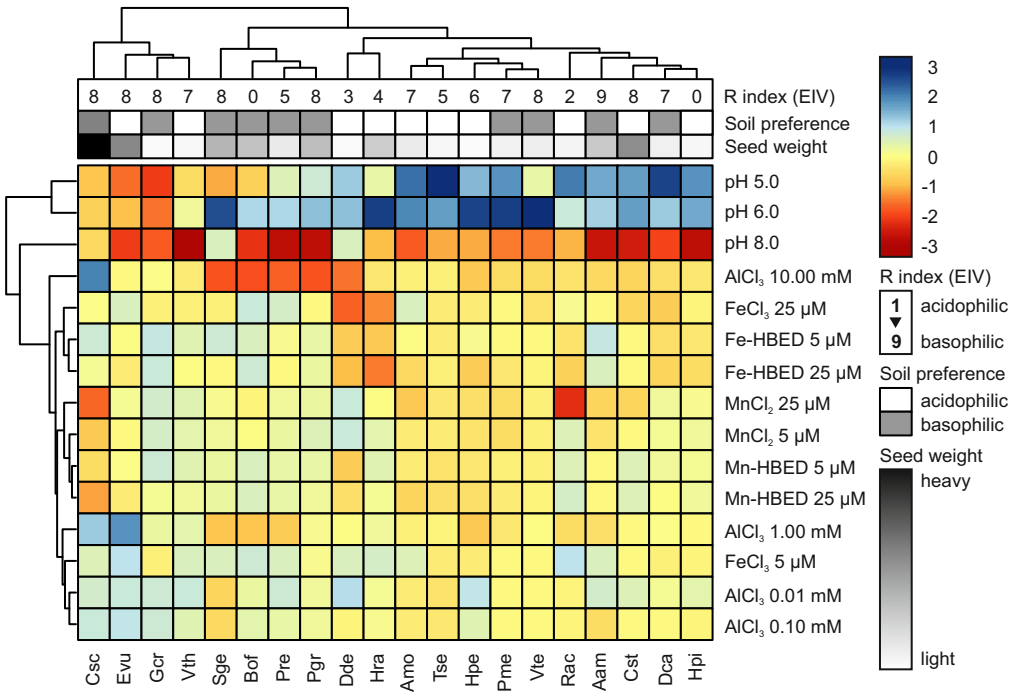

Figure 5 Hierarchical clustering analysis (HCA) of germination reactions (FGP) of the studied species to the tested conditions. Analysis was conducted using data from Experiments 1–4. For each species, the data were normalized to FGP values recorded at pH = 7 (Experiment 1) or control (null concentration) conditions (Experiments 2–4). Three levels of annotations represents data presented in Table 1 (R Ellenberg's Indicator Value, estimated soil preference and seed weight). Blue and red colors in scale bar represents stimulation and inhibition of FGP, respectively. Phenograms were constructed using Ward's method of row clustering and Manhattan distance.

Full-size DOI: 10.7717/peerj.13255/fig-5

The recorded data clearly demonstrated that ability of the studied species to complete germination was pH-dependent (hypothesis 1). Even the species considered as undeniable acidophiles and basophiles (considering their centers of abundance) are able to complete germination under the conditions which are not their typical ones (*e.g.*, Aam, Bof, Csc, Cst, Dca, Dde, Evu, Pre, Pgr, Rac, Vth and Vte). Furthermore, for some species that can be found both in xerothermic and dry grasslands (Cst, Dca, Hpe, and Evu) the amplitude of soil pH allowing them completion of germination is relatively wide. However, it should be interpreted with caution because mismatch between the conditions allowing completion of germination and those permitting optimal vegetative growth are known, which was shown previously, *e.g.*, for soil moisture (*Wilman et al., 2014*). Overall, it is worth noting that both alkaline xerothermic and acidic dry grasslands are composed of (1) species with pH requirements for completion of germination exactly matching the pH of the substratum on which they can be found, (2) pH-undifferentiated species (wide theoretical regeneration niche) and (3) species characterized with marked pH preferences however having also partial ability to complete germination under non-optimal conditions. It is known that there is diversity of germination strategies within the flora of alkaline and acidic grasslands, including such adaptations as reaction to nitrogen (*Wala, Kołodziejek & Patykowski, 2021*),

ability to withstand thermal aging (*Tausch et al., 2019*) and complete germination under a given thermoperiod (*Tudela-Isanta et al., 2017*). Although previous studies suggested that alpine calcicoles and calcifuges possessed group-specific adaptations allowing completion of germination under acidity matching their centers of abundance (*Tudela-Isanta et al., 2018*), a homogeneous reaction within a group is probably not as plausible in lowland temperate grasslands (*Margreiter et al., 2020*). Our study is in agreement with the conception of heterogeneity of germination strategies within calcicole/calcifuge groups, as it clearly shows that a potential germination-based regeneration niche of several species (*e.g.*, Aam, Cst, Dde, Pgr, Rac) is wide and does not match their realized niches and centers of abundance. These species cannot be usually found under non-typical soil conditions, which probably results from weak competitiveness at the vegetative stage. For example, Rac is known to be a pH-indifferent germinator (*Yazdi et al., 2013*), but is not able to win competition with basophilic species during establishment on calcareous soils, also due to susceptibility to Fe-dependent limitations (*Hutchinson, 1967*; *Leuchner & Ellenberg, 2017*). On the other hand, some calcicole species (occurring naturally on alkaline soils, *e.g.*, Rendzinas) showed exceptional tolerance to low pH at the germination stage (*e.g.*, Aam and Pgr) but they were not able to develop correctly under high soil acidity of Podzols (*Wala et al., 2020*).

It must be noted that the composition of buffer solution used for studies on pH optimum for completion of germination can affect the results (*Ma et al., 2015*). Due to the fact that we used in this study isomolar buffers containing only phosphates, the results can be the effect of any of the three traits of the used buffers, namely differing acidity (pH) or phosphate ratio (HPO_4^{2-}: $H_2PO_4^-$) or ratio of Na^+: K^+. Thus, species limited by the highest pH values used in this study (calcicole Aam, Amo, Bof, Pre, Pgr and Pme and calcifuge Hpi) may also additionally encounter adverse effects of HPO_4^{2-} and/or Na^+. As both phosphate forms can be utilized by plants; *Schachtman, Reid & Ayling, 1998*), Na-caused toxicity is more probable explanation. This element (whose availability can be increased due to agronomical nutrient loadings; *Núez-Delgado, López-Periago & Díaz-Fierros Viqueira, 2002*) can disturb the germination-based revegetation of the studied types of grasslands because it is relocated with percolate during soil surface runoff across slopes (*Schulze et al., 2019*) from arable fields to lower areas. Sodic-saline conditions were repeatedly shown to deteriorate edaphic conditions, ability of seeds to complete germination, plant performance (*Läuchli & Grattan, 2012*) and regeneration sites (*Ma et al., 2015*). Therefore, it can be treated as another threat for the species we studied. Additionally, it is worth noting that alkaline soils, on which xerothermic grasslands are settled in Central Europe, are rather non-sodic (*Tóth, Montanarella & Rusco, 2008*). It partially explains why the studied xerothermic calcicoles are probably not adapted to elevated Na concentrations and can suffer from adverse effects of this element. It provokes further studies concerning the effects of sodic-alkaline environment on the ability of seeds to complete germination.

Influence of Fe on the ability of seeds to complete germination was only occasionally studied (*El Rasafi et al., 2016*; *Reis et al., 2018*). For example, 20 μmol dm^{-3} solution of $FeCl_3$ was shown to have no significant effect on the ability to complete germination of crop plants such as *Triticum aestivum* L. and *Phaseolus vulgaris* L. (*El Rasafi et al., 2016*) and similar reactions were observed for Bof, Csc, Cst and Tse investigated in this study.

On the other hand, a reduction of FGP due to application of ionic Fe (given as $FeSO_4$) was observed in *T. aestivum* treated with solutions of c.a. 90–143 μmol $Fe^{2+} \cdot dm^{-3}$ (*Reis et al., 2018*). Interestingly, there are also some available data suggesting that completion of germination in rice (*Oryza staviva* L.) could be accelerated due to application of $FeSO_4$ (c.a. 6.6 mmol $Fe^{2+} \cdot dm^{-3}$; *Wang et al., 2020*). In the presented study, detrimental changes pertaining to germination parameters due to application of $FeCl_3$ could be the result of Fe itself or Cl^- load (15/75 μmol $Cl^- \cdot dm^{-3}$). However, it is worth noting that due to low concentrations of the tested solutions, effects of osmotic pressure are not high enough to affect germination at imbibition phase, which can be extrapolated from numerous studies showing effects of polyethylene glycol-simulated osmoticum on the ability of seeds to complete germination (*Chamorro & Moreno, 2019*; *Yi et al., 2019*). This strongly suggests that several studied species (Aam, Dca, Dde, Gcr, Hpi, Hra, Pgr, Vth and Vte) are susceptible to overdose of Fe^{3+}, as considerable concentrations of Cl^- (up to 30 mmol dm^{-3}) are known to have no or marginal influence on the germination process (*Abedi, Bartelheimer & Poschlod, 2013*). Although mechanism of Fe toxicity still needs detailed studies, it can be hypothesized that reduced completion of germination due to supraoptimal doses of Fe can be the result of Fe-dependent disturbances in a cell cycle (*Reis et al., 2018*) resulting from oxidative stress caused by photo-Fenton and Fenton-like reactions catalyzed by Fe (*Rincón & Pulgarin, 2006*; *Ahile et al., 2021*) and/or non-oxidative ion-specific toxicity. In nature, release of Fe species from Fe-bearing minerals is to a great extent controlled by soil pH (*Strawn, Bohn & O'Connor, 2020*). In fact, concentration of Fe^{3+} released from Fe oxides under alkaline conditions (pH = 8.5) is very low and becomes significant only at remarkably acidic conditions (*Robin et al., 2008*). It partially explains why some undeniable calcicoles (Aam, Dca, Gcr, Pgr, Sge, and Vte) are negatively influenced by ionic Fe. However, some studied calcifuges (Cst, Dde, Hpi, Hra and Vth) also seem to be prone to Fe-dependent limitations at the germination stage (hypothesis 2). It can be proposed that Fe soil status is an additional but minor ecological sieve filtering establishment of new individuals in the studied communities. However, the way in which Fe becomes a potential limiting factor in each of the studied types of communities may vary. For example, in the case of dry acidic grasslands, availability of iron can be shaped by succession (*Sparrius, Sevink & Kooijman, 2012*). Another way to increase availability of Fe on soil surface is severe soil disturbance (caused by anthropogenic and natural activities) and eventually displacement of Fe-rich hardpan, which is then solubilized. A different mode of action is more plausible in alkaline xerothermic grasslands, where Fe chelation due to biological activity at the local scale is probably the major factor influencing its availability (*Singer, Schwertmann & Friedl, 1998*; *Gries & Runge, 1992*; *Ferreira et al., 2019*). Although not much is known about the quantitative siderophoral status of Rendzinas, studies on Podzols showed that this kind of soil contains nanomolar concentrations of a given siderophore/chelator (a dozen or so nanomoles on average; *Winkelmann, 2007*; *Ahmed & Holmström, 2015*). Thus, it is very likely that the total pool of Fe chelators (and thus chelated Fe as there is a linkage between concentration of chelators and chelated Fe; *Liu & Millero, 1999*) is at the micromolar level in both soils (*Robin et al., 2008*), but their biological recovery from the alkaline soil may be even harder than from the acidic one in

some cases due to lime-dependent sequestration (*Boiteau et al., 2020*). In the context of the presented study, such chelator-based Fe dissolution in soil may have some impact on germination-based revegetation, affecting the studied plants in a species-specific manner.

The role of ionic and complexed forms of Mn on geochemical processes and life (including plant ontogenesis, *e.g.*, a germination process) still needs elucidation (*Duckworth, Bargar & Sposito, 2009*). To date, it has been demonstrated that Mn (solutions of c.a. 5–91 μmol $Mn^{2+} \cdot dm^{-3}$ given as $MnSO_4$) did not significantly affect FGP of lettuce (*Lactuca sativa* L.) seeds sown on Hoagland medium (*Liu, Zhang & Lal, 2016*). On the other hand, the completion of germination of *Nicotiana tabacum* L. seeds was significantly reduced up to 75% after application of 2–20 mmol $Mn^{2+} \cdot dm^{-3}$ (given as $MnSO_4$) on Murashige and Skoog medium (*Santandrea, Schiff & Bennici, 1997*). Reduction of FGP due to increasing availability of Mn seems to be species-specific, as plants can differ in their reaction to Mn concentration at the germination stage (*e.g.*, *Arabidopis thaliana* L. and *Arabis paniculata* Franch. subjected to 1 and 10 mmol $Mn^{2+} \cdot dm^{-3}$ given as $MnCl_2$; *Tang, Tao & Li, 2021*). On the basis of the presented study it can be stated that Mn species-specifically influenced the ability of seeds to complete germination (hypothesis 2). As eight studied species did not suffer from the negative effects of Mn (Csc, Dca, Gcr, Hpi, Pre, Tse, Vth and Vte) and there was no sharply pronounced pattern of reaction to the tested stimuli within each ecological group, it can be proposed that the reaction to Mn availability is individual for each species, which further implies that microhabitats (in terms of Mn availability gradient or hotspots) can play a role in establishment of the studied plants. It is however worth noting that oxidative state of Mn also should not be underestimated, as chemical structure HBED is believed to favor Mn(III) state (similarly to other Mn chelators; *Duckworth, Bargar & Sposito, 2009*) due to two phenolato donors of this chelator (*Pinto et al., 2019*) and/or oxidation under oxygen environment (*Wahsner et al., 2019*), although occurrence of Mn(II) state is also possible. From an environmental point of view, it is believed that complexed forms of Mn prevail in soil solution at neutral pH (>90% of Mn pool), while under acidic conditions, free Mn^{2+} is probably the predominant form of this element (>70% of Mn pool; *Ritchie, 1989*). Slightly reduced FGP and IGV values in basophilic species (Aam, Bof, Csc, Pme, Pgr and Sge) treated with Mn-HBED as well as in acidophilic ones (Amo, Evu, Hpe, Hra and Rac) treated with $MnCl_2$ suggest that the predominant form of Mn in each type of grasslands plays a role in assembly of plant community.

Interplay between endogenous Fe and Mn is known to influence the seed germination process (*Eroglu et al., 2017*). The present study showed that exogenous Fe and Mn also affected the ability of seeds to complete germination. Additionally, considering the possible application of our results, it seems that the chelated forms of the studied metals (Fe-HBED and Mn-HBED) are not more detrimental to the germination process in the studied plants when compared to their respective ionic forms (isomolar solutions). This indicates that reasonable usage of HBED-chelated Fe and Mn should not pose a risk to terrestrial vegetation, which was suggested for other Fe chelates (*European Food Safety Authority, 2013*).

Limitations caused by increased Al availability were widely shown to occur in many terrestrial plant species (*Singh, 2017*). Inhibition of root elongation caused by

destabilization of cell homeostasis is the most recognized sign of Al toxicity at the early stages of plant growth (*Alves Silva et al., 2014*). However, relatively little is known about the direct influence of Al on the ability of seeds to complete germination. To date, the most detailed study on this topic has been conducted on plants from dry temperate grasslands that can be found along an acidity gradient (*Abedi, Bartelheimer & Poschlod, 2013*). Among the 15 investigated species, a little more than half of them were resistant to Al toxicity at the germination stage (which was also shown for Amo, Cst, Dca, Rac, Tse and Vth investigated in this study), but for the other near half Al was toxic when the seeds were treated with its concentrations exceeding 1–2 mmol dm^{-3} (*Abedi, Bartelheimer & Poschlod, 2013*). Reduction of completion of germination (by c.a. 25%) due to increased availability of Al^{3+} (320 μmol dm^{-3}) was also observed in *Ricinus communis* L. (*Alves Silva et al., 2014*) as well as in *Eugenia dysenterica* DC. (200–800 μmol $Al^{3+} \cdot dm^{-3}$; reduction by 30%; *Rodrigues et al., 2019*). On the other hand, *L. sativa* is known to complete germination in the presence of Al within the range of c.a. 2–800 μmol $Al^{3+} \cdot dm^{-3}$, but concentrations higher that 2 μmol $Al^{3+} \cdot dm^{-3}$ slow down this process (*Silva & Matos, 2016*). Decelerated germination was also observed in *E. dysenterica* subjected to 600–800 μmol $Al^{3+} \cdot dm^{-3}$ (*Rodrigues et al., 2019*). Similar mode of action was also noted in this study, but the thresholds for the observed effect were species-specific. For example, undeniable basophilic species (Aam, Pgr and Sge) together with Dde (acidophilic species) were susceptible to Al toxicity even at its low concentrations, whereas the reduction of completion of germination for some other species was observed in 1 mmol $Al^{3+} \cdot dm^{-3}$ (Bof, Hpi, Pme and Pre) and in 10 mmol $Al^{3+} \cdot dm^{-3}$ (Evu, Gcr, Hra and Vte). It suggests that Al can be a barrier preventing establishment of basophiles on non-alkaline soils, whilst for acidophiles it probably contributes to the formation of a mosaic structure of vegetation. It must be noted however that slight acidification events in alkaline xerothermic grasslands probably do not lead to strong Al-driven shifts in germination-dependent revegetation, as basophilic species are able to complete germination efficiently under low Al concentrations. Furthermore, the basophiles seemed not to be more susceptible to Al toxicity than the tested acidophiles (hypothesis 3). It is known that the acquisition of Al is connected with generation of reactive oxygen species leading to oxidative stress and cellular damages (*Šimonovičová et al., 2004*; *Bothe, 2015*). It can be hypothesized that these processes occur also in a growing embryo when seeds encounter high amounts of freely available Al during imbibition. However, there are also some suggestions (*Silva & Matos, 2016*) that morpho-anatomical and chemical traits of a seed coat contribute to Al resistance at the germination stage, probably because of limited penetration of this element due to chemical constituents of this structure (most notably phenolic compounds that are able to complex Al^{3+} into insoluble forms; *Moïse et al., 2005*; *Zhang et al., 2016*). Alternatively, germinating seeds of tolerant species are able to withstand Al-caused toxicity on account of efficient stress coping mechanisms that reduce ion-specific toxicity and oxidative stress (resulting in fast compensation of damages). As many species in our investigation completed germination under high concentrations of Al, their tolerance buffer for completion of germination is wider than it could be predicted from their centers of abundance (*Abedi, Bartelheimer & Poschlod, 2013*). However, further studies are needed to determine if the tolerance level at the germination stage matches that

at further ontogenetic stages, up to maturity (including reproduction). It can be predicted that if the tolerance buffer to Al toxicity during germination of a given species is greater than those during further developmental stages (*e.g.*, growth of seedlings), depletion of seed banks is very plausible, which was proposed for other environmental stimuli, *e.g.*, nitrogen (*Bird & Choi, 2017*).

Interestingly, some species may even experience positive effects of increased availability of Al, *e.g.*, remarkably acidophilic grass, *Corynephorus canescens* L. (most markedly at 0.01 mmol $Al^{3+} \cdot dm^{-3}$, but possibly up to 2 mmol $Al^{3+} \cdot dm^{-3}$; *Abedi, Bartelheimer & Poschlod, 2013*). Similar slight stimulation was observed in Hpe and Csc (0.01 and 10 mmol $Al^{3+} \cdot dm^{-3}$, respectively) investigated in this study. Enhanced completion of germination may be in this case the result of an increased pro-oxidative state due to Al-dependent stimuli (*Šimonovičová et al., 2004*), as reactive oxygen species (most notably hydrogen peroxide) are known to trigger the release from dormancy and accelerate remobilization of resources and endosperm weakening (*Wojtyla et al., 2016*). It can be proposed that such stimulation plays a role in detection of optimal conditions for growth and development in some Al-tolerant specialists (in this case Hpe and Csc).

Finally, the presented study showed that flora from both acidic dry and alkaline xerothermic grasslands was diverse in its germination strategies. Hierarchical clustering analysis indicated that there was no homogenous reaction to the tested conditions within each group. Furthermore, seed size and estimated preference for soil acidity should not be used as common delimiters allowing sharp segregation of germination strategies of acidophilic and basophilic plants (in terms of influence of edaphic conditions, *e.g.*, pH, Al, Fe and Mn). Moreover, seed weight did not correlate with the ability of seeds to complete germination under increased availability of the tested metals or changing acidity (with sparse exceptions). This suggests the existence of some more complicated species-specific structural and/or physiological adaptations in seeds that are probably not yet well recognized to be the traits associated with tolerance to chemical stimuli.

CONCLUSION

Overall, the present study showed a diversity of germination strategies in plants from acidic dry as well as alkaline xerothermic grasslands. Tolerances to pH and availability of Fe, Mn and Al are other traits contributing to floristic diversity of a given plant community due to the formation of microhabitats. This seems to promote mosaic structure of the studied plant communities; however; in our opinion pH and the extremely high availability of Al are major factors, while availability of Fe and Mn is probably of secondary importance. The studied edaphic factors seem to filter plant establishment on remarkably acidic and alkaline soils and might be among the driving forces of evolution of plant germination strategies. As specialization of germination requirements in the studied species was found, changes in soil pH and availability of Al, Fe and Mn should be taken into consideration in further studies on biology of dry acidic, and xerothermic alkaline grasslands. It can be expected that spatial and temporal changes in local vegetation have an impact on germination-based revegetation in the studied types of communities. Thus, we believe that

ecology of acidic and alkaline grasslands should be co-investigated with soil chemistry. Such studies cannot only deepen understanding of species diversity and natural processes in these kinds of communities (*e.g.*, succession), but also can improve management, protection and ecological restoration of grasslands in a worldwide scale. Furthermore, both Fe-HBED and Mn-HBED exerted similar effects on the ability of seeds to complete germination when compared to ionic forms of these metals. This suggests that both chelates are not detrimental to early ontogenetic stages of plants when they are reasonably used.

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Competing Interests

Tomasz Wilk is an employee of PPC ADOB and was responsible for synthesis of Mn-HBED.

Author Contributions

- Mateusz Wala conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Jeremi Kołodziejek conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Tomasz Wilk performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

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The following patent dependencies were disclosed by the authors:

European Patent Application EP20461587.6 (A PROCESS FOR THE PREPARATION OF SALTS OF N,N'-DISUBSTITUTED ETHYLENEDIAMINE-N,N'-DIACETIC ACID DERIVATIVES AND THEIR USE; Date of filling: 01.12.2020)

Data Availability

The following information was supplied regarding data availability:

The raw measurements (values of final germination percentage and index of germination velocity) are available in the Supplementary File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.13255#supplemental-information.

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P–4

Materiał uzupełniający

(Supplementary material)

Supplemental Table 1. Acidity of the tested Fe-, Mn- and Al-containing solutions presented as pH values. The pH value of water used for control variants and for preparation of the tested solutions was 5.81.

Concentration of tested solution		pH value
$FeCl_3$ [µmol · dm^{-3}]	5	5.10
	25	4.37
Fe-HBED [µmol · dm^{-3}]	5	5.75
	25	5.74
$MnCl_2$ [µmol · dm^{-3}]	5	5.40
	25	5.31
Mn-HBED [µmol · dm^{-3}]	5	5.82
	25	5.99
$AlCl_3$ [mmol · dm^{-3}]	0.01	5.38
	0.10	4.41
	1.00	3.89
	10.00	3.69

Supplemental Table 2. Results of two-way ANOVA (*F* values and significance) showing influence of the studied factors (type and dose of the tested compounds) and their interactions on final seed germination percentage (FGP) and index of germination velocity (IGV) of the studied species. [a]$FeCl_3$ or Fe-HBED/$MnCl_2$ or Mn-HBED; [b]5 or 25 µmol · dm^{-3}. Differences considered as statistically significant ($p < 0.05$) were bolded.

Species	*F* values and significance					
	Experiment 2			Experiment 3		
	Fe type[a] (FeT, *df* = 1)	Fe dose[b] (FeD, *df* = 1)	FeT x FeD (*df* = 3)	Mn type[a] (MnT, *df* = 1)	Mn dose[b] (MnD, *df* = 1)	MnT x MnD (*df* = 3)
	FGP					
Amo	272.3 (**<0.001**)	21.1 (**<0.001**)	45.7 (**<0.001**)	1.0 (0.343)	53.3 (**<0.001**)	1.9 (0.192)
Aam	22.1 (**<0.001**)	22.1 (**<0.001**)	2.5 (0.143)	7.4 (**0.019**)	0.5 (0.510)	0.5 (0.510)
Bof	0.7 (0.427)	0.7 (0.427)	0.2 (0.631)	21.4 (**<0.001**)	0.2 (0.682)	0.2 (0.682)
Csc	1.0 (0.327)	12.8 (**0.004**)	0.3 (0.619)	4.4 (0.058)	14.3 (**0.003**)	0.2 (0.682)
Cst	0.2 (0.663)	0.2 (0.663)	0.2 (0.663)	1.0 (0.337)	0.1 (0.745)	0.1 (0.745)
Dca	0.2 (0.646)	5.6 (**0.036**)	0.9 (0.364)	0.2 (0.657)	0.2 (0.657)	0.8 (0.381)
Dde	4.3 (0.061)	64.5 (**<0.001**)	44.3 (**<0.001**)	34.4 (**<0.001**)	0.4 (0.545)	0.4 (0.545)
Evu	22.1 (**<0.001**)	3.0 (0.109)	0.1 (0.809)	0.2 (0.648)	0.1 (0.878)	0.6 (0.450)
Gcr	82.3 (**<0.001**)	0.1 (0.828)	0.1 (0.828)	1.3 (0.274)	2.0 (0.177)	3.0 (0.111)
Hpi	13.8 (**0.003**)	1.2 (0.297)	1.2 (0.297)	0.1 (0.721)	0.1 (1.000)	0.5 (0.479)
Hpe	0.8 (0.396)	26.0 (**<0.001**)	22.5 (**<0.001**)	0.1 (0.847)	1.0 (0.343)	0.1 (0.847)
Hra	22.0 (**<0.001**)	65.6 (**<0.001**)	18.2 (**0.001**)	1.1 (0.306)	7.1 (**0.020**)	0.3 (0.603)
Pme	3.2 (0.099)	4.6 (0.053)	3.2 (0.099)	32.7 (**<0.001**)	0.2 (0.668)	0.1 (1.000)
Pre	19.2 (**<0.001**)	0.1 (0.721)	0.5 (0.479)	0.5 (0.512)	0.1 (0.825)	0.1 (0.825)
Pgr	5.2 (**0.041**)	0.8 (0.396)	0.3 (0.607)	1.3 (0.284)	2.2 (0.161)	0.1 (0.715)
Rac	9.3 (**0.010**)	2.6 (0.132)	0.8 (0.387)	17.0 (**0.001**)	14.2 (**0.003**)	17.0 (**0.001**)
Sge	1.5 (0.242)	37.9 (**<0.001**)	0.3 (0.607)	1.3 (0.272)	0.2 (0.631)	0.2 (0.631)
Tse	0.9 (0.358)	0.1 (1.000)	0.1 (0.755)	2.0 (0.178)	1.1 (0.305)	0.1 (1.000)
Vth	0.1 (0.801)	15.0 (**0.002**)	0.6 (0.454)	0.1 (0.773)	0.1 (0.773)	0.3 (0.566)
Vte	4.1 (0.065)	8.6 (**0.013**)	1.3 (0.282)	0.4 (0.565)	0.4 (0.565)	0.1 (0.847)
	IGV					
Amo	309.5 (**<0.001**)	24.1 (**<0.001**)	58.5 (**<0.001**)	1.3 (0.269)	53.8 (**<0.001**)	2.5 (0.143)
Aam	41.3 (**<0.001**)	15.5 (**0.002**)	1.0 (0.344)	16.4 (**0.002**)	1.1 (0.314)	0.7 (0.404)
Bof	1.2 (0.296)	1.1 (0.312)	0.1 (0.915)	23.2 (**<0.001**)	0.7 (0.422)	0.1 (1.000)
Csc	0.1 (0.724)	15.4 (**0.002**)	0.1 (0.782)	4.2 (0.063)	13.5 (**0.003**)	0.2 (0.700)
Cst	7.1 (**0.020**)	1.3 (0.282)	1.2 (0.303)	1.3 (0.286)	0.6 (0.470)	0.3 (0.576)
Dca	0.3 (0.581)	5.8 (**0.033**)	0.7 (0.430)	0.8 (0.399)	0.5 (0.474)	1.0 (0.346)
Dde	3.7 (0.079)	70.1 (**<0.001**)	43.3 (**<0.001**)	35.5 (**<0.001**)	0.2 (0.644)	1.0 (0.339)
Evu	28.7 (**<0.001**)	4.0 (0.069)	0.5 (0.492)	0.2 (0.650)	0.1 (0.723)	0.6 (0.446)
Gcr	75.1 (**<0.001**)	0.1 (0.787)	2.8 (0.117)	1.6 (0.233)	3.0 (0.109)	5.1 (**0.043**)
Hpi	11.3 (**0.006**)	4.0 (0.068)	1.6 (0.232)	0.2 (0.636)	0.1 (0.834)	0.1 (0.811)
Hpe	19.9 (**<0.001**)	16.6 (**0.002**)	32.1 (**<0.001**)	3.2 (0.101)	3.4 (0.090)	1.0 (0.345)
Hra	33.3 (**<0.001**)	74.3 (**<0.001**)	20.6 (**<0.001**)	0.9 (0.358)	6.0 (**0.030**)	0.5 (0.485)
Pme	2.9 (0.114)	6.2 (**0.029**)	3.3 (0.095)	30.5 (**<0.001**)	0.8 (0.391)	0.3 (0.620)
Pre	19.5 (**<0.001**)	0.1 (0.938)	0.4 (0.556)	0.3 (0.625)	0.1 (0.765)	0.1 (0.848)
Pgr	14.6 (**0.002**)	0.7 (0.435)	0.3 (0.586)	3.1 (0.102)	2.2 (0.164)	0.3 (0.592)
Rac	13.2 (**0.003**)	5.6 (**0.036**)	1.8 (0.210)	15.1 (**0.002**)	10.0 (**0.008**)	19.7 (**<0.001**)
Sge	0.6 (0.444)	34.2 (**<0.001**)	0.8 (0.395)	1.4 (0.253)	0.1 (0.755)	0.5 (0.500)
Tse	1.0 (0.339)	0.1 (0.914)	0.1 (0.929)	2.3 (0.154)	0.8 (0.377)	0.1 (0.966)
Vth	0.9 (0.371)	20.0 (**<0.001**)	2.2 (0.162)	0.4 (0.539)	0.3 (0.572)	0.4 (0.518)
Vte	4.3 (0.060)	7.9 (**0.016**)	3.7 (0.079)	0.1 (0.732)	0.5 (0.499)	0.1 (0.827)

Supplemental Table 3. Comparison of effects of isomolar solutions of tested metals (Me) on the ability of the seeds to complete germination. Data are presented as FGPs ratio between values recorded in Experiment 2 (effect of chelated and ionic Fe) and values recorded in Experiment 3 (effect of chelated and ionic Mn), where Me means Fe and Mn. Comparison was conducted using raw FGPs values (two-way ANOVA followed by Bonferroni's post-hoc test; n = 4). Differences considered as statistically significant (p <0.05) were bolded.

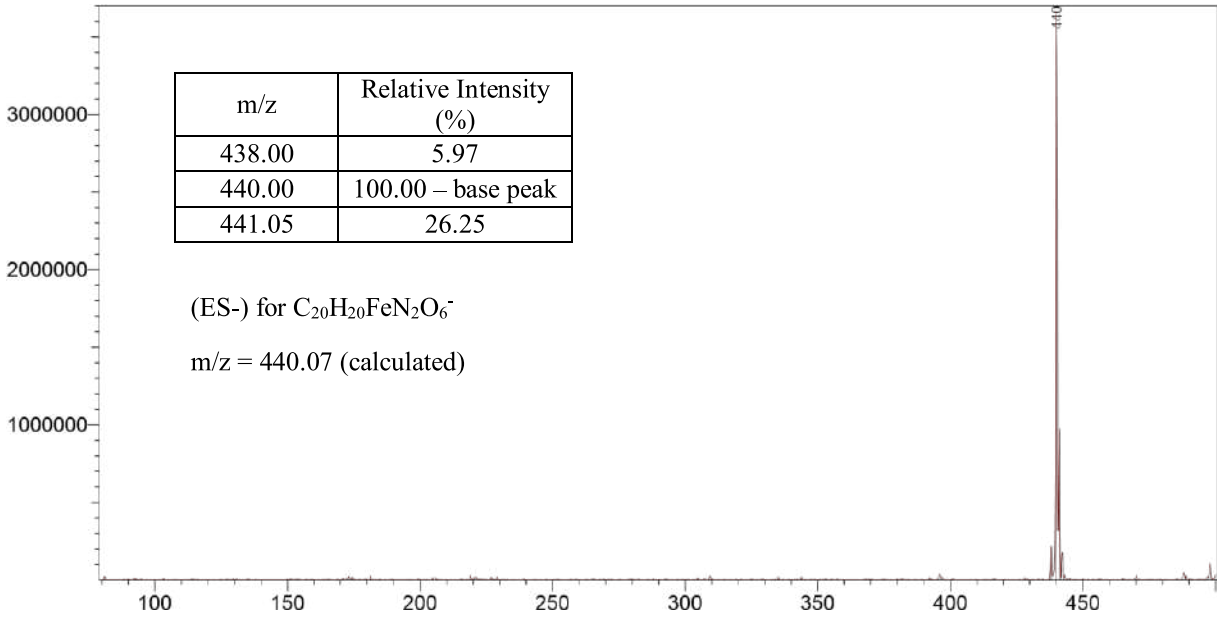
Species	$MeCl_X$		Me-HBED	
	5 µmol · dm^{-3}	25 µmol · dm^{-3}	5 µmol · dm^{-3}	25 µmol · dm^{-3}
Amo	1.317 (**<0.001**)	2.122 (**<0.001**)	1.048 (1.000)	0.936 (1.000)
Aam	1.429 (**0.002**)	1.263 (0.108)	1.375 (**0.001**)	1.250 (**0.023**)
Bof	1.385 (**0.025**)	1.486 (**0.007**)	1.020 (1.000)	1.102 (1.000)
Csc	1.783 (**0.001**)	2.692 (**<0.001**)	1.630 (**<0.001**)	1.895 (**<0.001**)
Cst	1.010 (1.000)	1.010 (1.000)	1.000 (1.000)	1.000 (1.000)
Dca	0.968 (1.000)	0.884 (**0.037**)	0.926 (0.159)	0.924 (0.159)
Dde	1.000 (1.000)	0.522 (**<0.001**)	1.043 (1.000)	0.900 (0.962)
Evu	1.250 (**0.044**)	1.130 (0.653)	1.038 (1.000)	1.040 (1.000)
Gcr	0.863 (0.240)	0.852 (0.164)	1.098 (0.223)	1.254 (**0.001**)
Hpi	0.923 (0.456)	0.944 (1.000)	0.867 (**0.032**)	0.793 (**0.001**)
Hpe	1.123 (0.565)	1.145 (0.360)	1.293 (**0.004**)	0.855 (0.330)
Hra	1.125 (0.479)	0.595 (**<0.001**)	0.673 (**<0.001**)	0.533 (**<0.001**)
Pme	1.179 (0.115)	1.182 (0.115)	0.957 (1.000)	0.809 (**0.018**)
Pre	1.068 (1.000)	1.081 (0.693)	0.945 (1.000)	0.917 (0.680)
Pgr	0.815 (**0.009**)	0.816 (**0.015**)	0.909 (0279)	0.929 (0.732)
Rac	1.000 (1.000)	1.271 (**0.032**)	0.866 (**0.029**)	0.831 (**0.005**)
Sge	1.145 (0.247)	0.932 (1.000)	1.188 (0.107)	0.891 (0.815)
Tse	0.900 (0.843)	0.925 (1.000)	0.985 (1.000)	1.048 (1.000)
Vth	1.022 (1.000)	0.914 (0.367)	1.000 (1.000)	0.967 (1.000)
Vte	0.978 (1.000)	0.868 (**0.021**)	0.989 (1.000)	0.925 (0.378)

Supplemental Table 4. Results of three-way ANOVA (*F* values and significance) showing influence of the studied factors (metal, type and dose of the tested compounds) and their interactions on final seed germination percentage (FGP) and index of germination velocity (IGV) of the studied species. [a]Fe or Mn; [b]$MeCl_X$ or Me-HBED; [c]5 or 25 µmol · dm^{-3}. Differences considered as statistically significant ($p < 0.05$) were bolded.

Species	*F* values and significance						
	(M) Metal[a] (df = 1)	(T) Type of compound[b] (df = 1)	(D) Dose of compound[c] (df = 1)	M x T (df = 1)	M x D (df = 1)	T x D (df = 1)	M x T x D (df = 1)
	FGP						
Amo	110.7 (**<0.001**)	79.7 (**<0.001**)	74.1 (**<0.001**)	110.7 (**<0.001**)	10.2 (**0.004**)	8.2 (**0.008**)	26.0 (**<0.001**)
Aam	68.2 (**<0.001**)	23.4 (**<0.001**)	9.8 (**0.004**)	0.1 (0.778)	4.0 (0.058)	2.0 (0.167)	0.1 (0.778)
Bof	25.5 (**<0.001**)	4.8 (**0.038**)	0.2 (0.701)	12.2 (**0.002**)	0.8 (0.374)	0.4 (0.524)	0.1 (0.898)
Csc	144.1 (**<0.001**)	5.2 (**0.032**)	26.9 (**<0.001**)	0.9 (0.340)	0.4 (0.523)	0.1 (1.000)	0.4 (0.523)
Cst	0.3 (0.598)	1.1 (0.296)	0.3 (0.598)	0.3 (0.598)	0.1 (1.000)	0.3 (0.598)	0.1 (1.000)
Dca	21.0 (**<0.001**)	0.4 (0.519)	3.9 (0.061)	0.1 (1.000)	1.7 (0.203)	0.1 (1.000)	1.7 (0.203)
Dde	20.1 (**<0.001**)	36.2 (**<0.001**)	15.8 (**<0.001**)	13.8 (**0.001**)	24.9 (**<0.001**)	17.9 (**<0.001**)	10.2 (**0.004**)
Evu	10.0 (**0.004**)	8.4 (**0.008**)	1.1 (0.301)	4.5 (**0.045**)	0.6 (0.436)	0.3 (0.602)	0.6 (0.436)
Gcr	0.1 (0.721)	15.8 (**<0.001**)	1.8 (0.198)	34.8 (**<0.001**)	1.2 (0.289)	2.4 (0.131)	1.8 (0.198)
Hpi	36.3 (**<0.001**)	4.4 (**0.046**)	0.5 (0.491)	7.1 (**0.014**)	0.5 (0.491)	0.1 (0.890)	1.6 (0.220)
Hpe	9.9 (**0.004**)	0.3 (0.604)	19.9 (**<0.001**)	0.6 (0.439)	9.9 (**0.004**)	13.5 (**0.001**)	11.7 (**0.002**)
Hra	64.0 (**<0.001**)	9.0 (**0.006**)	64.0 (**<0.001**)	18.8 (**<0.001**)	21.8 (**<0.001**)	13.4 (**0.001**)	9.0 (**0.006**)
Pme	0.3 (0.584)	4.9 (**0.036**)	3.8 (0.064)	24.9 (**<0.001**)	1.9 (0.178)	1.9 (0.178)	1.9 (0.178)
Pre	0.1 (0.888)	14.7 (**<0.001**)	0.2 (0.674)	8.9 (**0.007**)	0.1 (0.888)	0.5 (0.485)	0.2 (0.674)
Pgr	36.2 (**<0.001**)	0.8 (0.379)	2.8 (0.109)	5.9 (**0.023**)	0.1 (0.704)	0.4 (0.528)	0.1 (0.899)
Rac	1.2 (0.275)	0.8 (0.394)	14.8 (**<0.001**)	25.9 (**<0.001**)	2.6 (0.120)	12.9 (**0.001**)	5.6 (**0.027**)
Sge	1.4 (0.241)	2.8 (0.106)	20.8 (**<0.001**)	0.1 (1.000)	14.8 (**<0.001**)	0.1 (1.000)	0.5 (0.478)
Tse	1.4 (0.246)	0.1 (0.814)	0.5 (0.482)	2.8 (0.109)	0.5 (0.482)	0.1 (0.814)	0.1 (0.814)
Vth	1.3 (0.261)	0.1 (0.899)	4.7 (**0.040**)	0.1 (0.704)	2.8 (0.109)	0.1 (0.899)	0.8 (0.379)
Vte	10.7 (**0.003**)	3.2 (0.087)	2.2 (0.151)	0.8 (0.382)	5.7 (**0.026**)	0.8 (0.382)	0.4 (0.558)
	IGV						
Amo	107.1 (**<0.001**)	71.9 (**<0.001**)	77.9 (**<0.001**)	108.9 (**<0.001**)	12.6 (**0.002**)	7.8 (**0.010**)	29.5 (**<0.001**)
Aam	64.8 (**<0.001**)	48.3 (**<0.001**)	9.4 (**0.005**)	0.1 (0.824)	1.8 (0.198)	1.6 (0.217)	0.1 (0.869)
Bof	23.8 (**<0.001**)	4.7 (**0.040**)	0.1 (0.767)	15.0 (**<0.001**)	1.8 (0.192)	0.1 (0.933)	0.1 (0.933)
Csc	143.9 (**<0.001**)	3.2 (0.086)	28.6 (**<0.001**)	1.7 (0.200)	0.1 (0.817)	0.1 (0.909)	0.2 (0.633)
Cst	17.7 (**<0.001**)	5.8 (**0.024**)	1.5 (0.226)	0.3 (0.594)	0.1 (1.000)	1.2 (0.294)	0.1 (0.911)
Dca	16.2 (**<0.001**)	1.0 (0.322)	5.1 (**0.033**)	0.1 (0.856)	1.6 (0.224)	0.1 (0.942)	1.6 (0.217)
Dde	17.4 (**<0.001**)	36.5 (**<0.001**)	17.4 (**<0.001**)	15.6 (**<0.001**)	24.7 (**<0.001**)	19.6 (**<0.001**)	7.6 (**0.011**)
Evu	14.0 (**0.001**)	11.1 (**0.003**)	2.0 (0.174)	6.5 (**0.018**)	0.6 (0.436)	0.1 (0.790)	1.1 (0.305)
Gcr	29.6 (**<0.001**)	29.6 (**<0.001**)	1.0 (0.336)	51.4 (**<0.001**)	1.9 (0.177)	7.7 (**0.010**)	0.1 (0.747)
Hpi	45.1 (**<0.001**)	3.0 (0.097)	1.2 (0.286)	6.2 (**0.020**)	2.0 (0.167)	0.4 (0.556)	1.0 (0.337)
Hpe	27.1 (**<0.001**)	19.2 (**<0.001**)	17.3 (**<0.001**)	3.4 (0.078)	2.3 (0.141)	21.7 (**<0.001**)	10.6 (**0.003**)
Hra	125.2 (**<0.001**)	14.3 (**<0.001**)	66.7 (**<0.001**)	25.2 (**<0.001**)	24.9 (**<0.001**)	15.4 (**<0.001**)	9.0 (**0.006**)
Pme	0.7 (0.405)	4.8 (**0.038**)	6.2 (**0.021**)	23.3 (**<0.001**)	1.8 (0.190)	1.2 (0.292)	3.0 (0.097)
Pre	0.1 (0.710)	15.0 (**<0.001**)	0.1 (0.809)	10.8 (**0.003**)	0.1 (0.909)	0.1 (0.710)	0.4 (0.551)
Pgr	55.6 (**<0.001**)	2.4 (0.131)	2.6 (0.122)	16.0 (**<0.001**)	0.2 (0.671)	0.6 (0.440)	0.1 (0.976)
Rac	4.1 (0.054)	0.1 (0.807)	15.3 (**<0.001**)	28.3 (**<0.001**)	0.4 (0.545)	16.8 (**<0.001**)	5.1 (**0.034**)
Sge	0.5 (0.483)	2.0 (0.167)	16.3 (**<0.001**)	0.2 (0.693)	12.6 (**0.002**)	1.2 (0.282)	0.1 (0.966)
Tse	1.5 (0.235)	0.2 (0.666)	0.4 (0.557)	3.2 (0.086)	0.6 (0.462)	0.1 (0.977)	0.1 (0.926)
Vth	0.1 (0.720)	0.1 (0.936)	7.5 (**0.011**)	1.0 (0.321)	3.0 (0.095)	0.1 (0.866)	1.8 (0.198)
Vte	13.4 (**0.001**)	2.5 (0.127)	1.5 (0.229)	1.1 (0.309)	5.4 (**0.030**)	1.9 (0.178)	1.1 (0.308)

Supplemental Table 5. Coefficients of correlation between relative response of seeds to the tested conditions and seed weight calculated using Speraman's sum rank test. Analysis was conducted using data from Experiments 1-4 for all tested species and for divided groups of acidophilous and basiphilous species. For each species, the data were normalized to FGP values recorded at pH = 7 (Experiment 1) or control (null concentration) conditions (Experiments 2-4) and weight of seeds were took from presented investigation (Table 1). Data is presented as coefficients with respective *p* values (showed in parentheses). Significant correlations were bolded ($p < 0.05$).

Conditions	Acidophilous species (n = 10)	Basophilous species (n = 10)	All species (n = 20)
	FGP		
Acidity (pH)			
5.0	-0,479 (0.162)	-0.358 (0.310)	-0.430 (0.058)
6.0	-0.309 (0.385)	-0.030 (0.934)	-0.200 (0.398)
8.0	0.127 (0.726)	0.055 (0.881)	0.023 (0.925)
Iron (Fe)			
5 µmol · dm^{-3} $FeCl_3$	0.139 (0.701)	0.248 (0.489)	0.090 (0.705)
25 µmol · dm^{-3} $FeCl_3$	0.333 (0.347)	-0.261 (0.467)	0.059 (0.803)
5 µmol · dm^{-3} Fe-HBED	-0.127 (0.726)	0.024 (0.947)	-0.050 (0.835)
25 µmol · dm^{-3} Fe-HBED	0.164 (0.651)	-0.224 (0.533)	0.051 (0.830)
Manganese (Mn)			
5 µmol · dm^{-3} $MnCl_2$	-0.164 (0.651)	-0.491 (0.150)	**-0.447 (0.048)**
25 µmol · dm^{-3} $MnCl_2$	-0.309 (0.385)	-0.539 (0.108)	-0.423 (0.063)
5 µmol · dm^{-3} Mn-HBED	0.297 (0.405)	-0.552 (0.098)	-0.206 (0.383)
25 µmol · dm^{-3} Mn-HBED	0.152 (0.675)	-0.285 (0.425)	-0.126 (0.598)
Aluminum (Al)			
0.01 mmol · dm^{-3}	-0.624 (0.054)	-0.505 (0.137)	**-0.510 (0.022)**
0.10 mmol · dm^{-3}	-0.188 (0.603)	-0.212 (0.556)	-0.173 (0.466)
1.00 mmol · dm^{-3}	0.454 (0.187)	-0.176 (0.627)	0.066 (0.782)
10.00 mmol · dm^{-3}	0.345 (0.328)	-0.309 (0.385)	-0.063 (0.791)
	IGV		
Acidity (pH)			
5.0	-0.430 (0.214)	-0.321 (0.365)	-0.415 (0.069)
6.0	-0.345 (0.328)	-0.067 (0.855)	-0.192 (0.416)
8.0	0.261 (0.467)	0.103 (0.777)	0.062 (0.796)
Iron (Fe)			
5 µmol · dm^{-3} $FeCl_3$	-0.309 (0.385)	0.406 (0.244)	-0.135 (0.571)
25 µmol · dm^{-3} $FeCl_3$	-0.042 (0.907)	-0.285 (0.425)	-0.102 (0.668)
5 µmol · dm^{-3} Fe-HBED	-0.188 (0.603)	0.285 (0.425)	-0.030 (0.900)
25 µmol · dm^{-3} Fe-HBED	0.176 (0.626)	-0.248 (0.489)	0.076 (0.750)
Manganese (Mn)			
5 µmol · dm^{-3} $MnCl_2$	-0.127 (0.726)	0.182 (0.614)	-0.363 (0.115)
25 µmol · dm^{-3} $MnCl_2$	-0.152 (0.676)	-0.134 (0.713)	-0.355 (0.125)
5 µmol · dm^{-3} Mn-HBED	0.333 (0.347)	0.115 (0.751)	-0.062 (0.796)
25 µmol · dm^{-3} Mn-HBED	0.309 (0.385)	0.358 (0.310)	0.002 (0.995)
Aluminum (Al)			
0.01 mmol · dm^{-3}	**-0.648 (0.043)**	-0.382 (0.276)	**-0.503 (0.024)**
0.10 mmol · dm^{-3}	-0.103 (0.777)	-0.152 (0.676)	-0.161 (0.498)
1.00 mmol · dm^{-3}	0.527 (0.117)	-0.079 (0.829)	0.191 (0.420)
10.00 mmol · dm^{-3}	0.418 (0.229)	-0.297 (0.405)	0.017 (0.945)

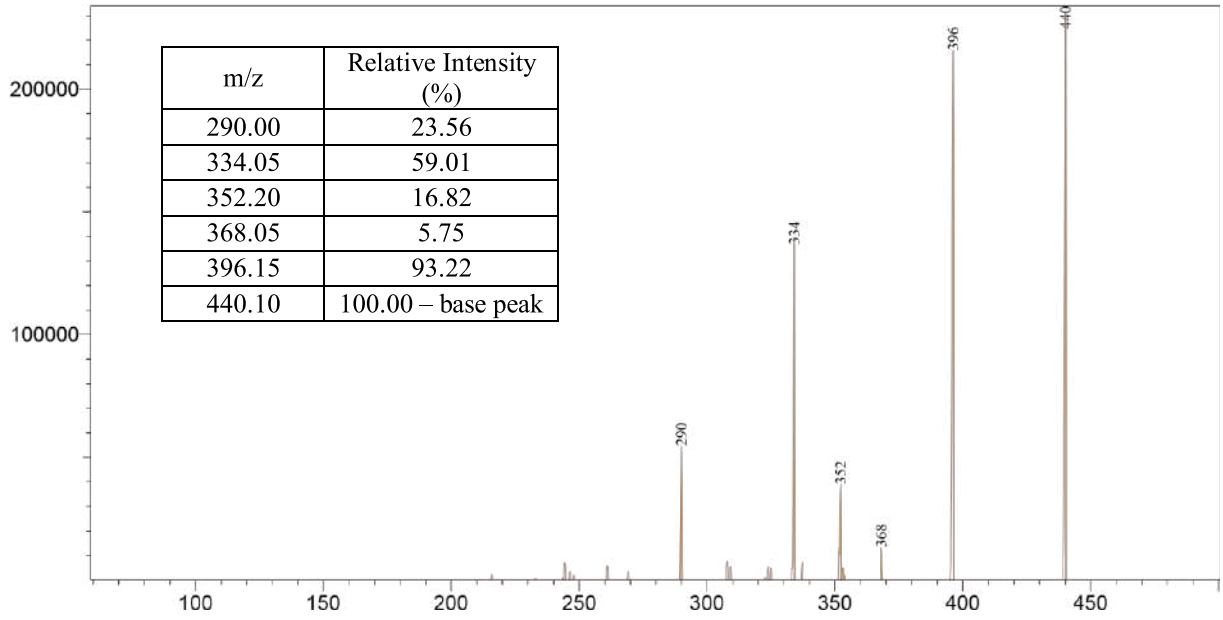


m/z	Relative Intensity (%)
438.00	5.97
440.00	100.00 – base peak
441.05	26.25

(ES-) for $C_{20}H_{20}FeN_2O_6^-$

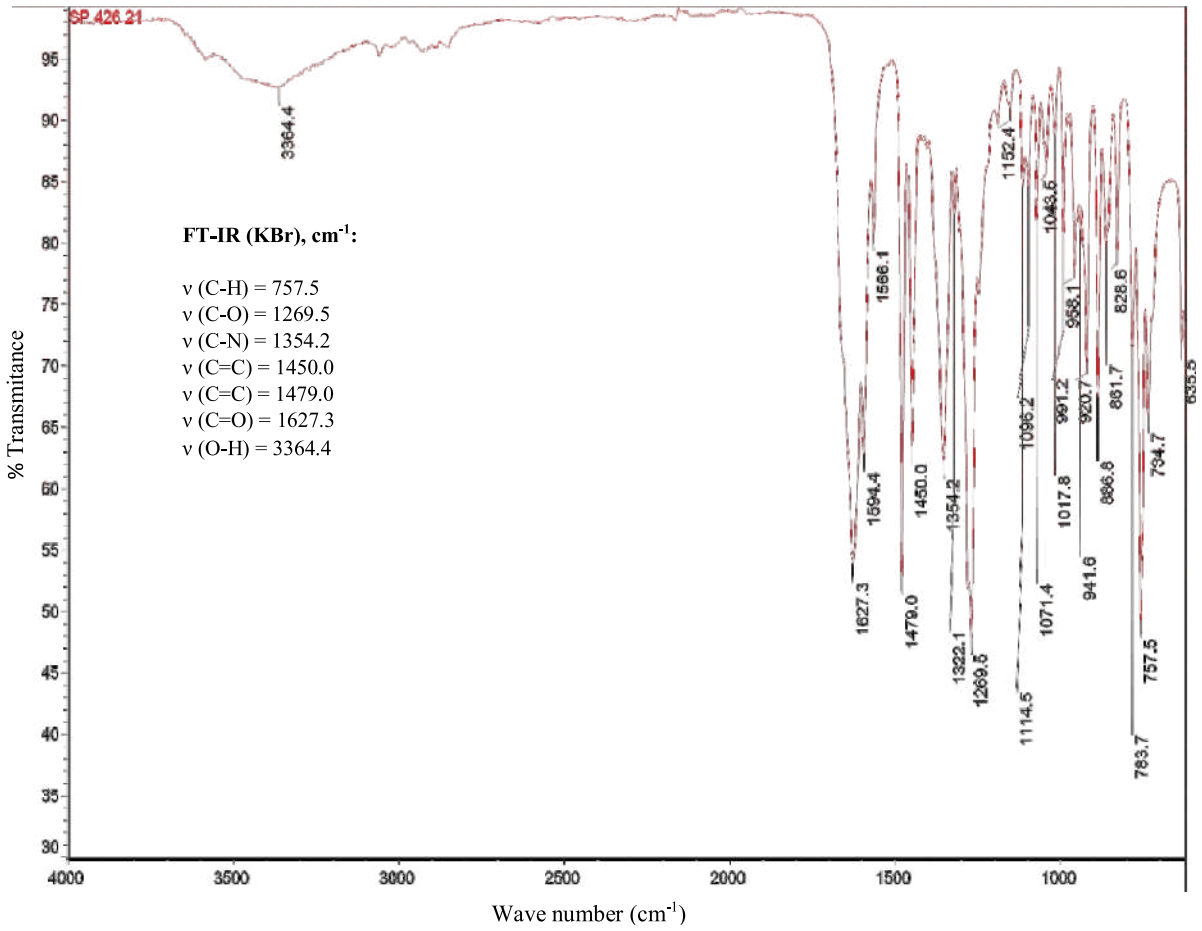
m/z = 440.07 (calculated)

A. LC-MS/MS spectrum (ES-) of *N*,*N*'-di(2-hydroxybenzyl)ethylenediamine-*N*,*N*'-diacetic acid iron(III) sodium salt, Fe-HBED.

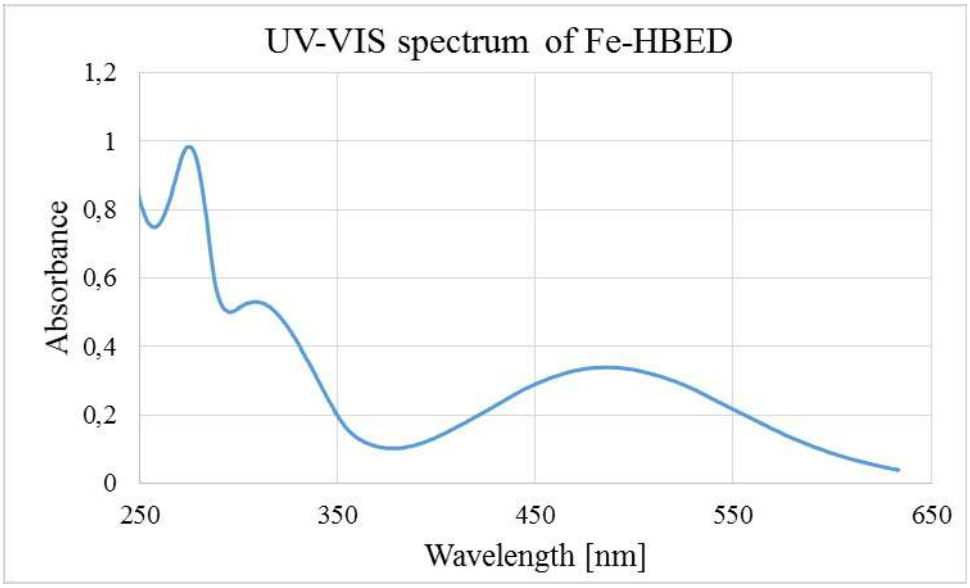


m/z	Relative Intensity (%)
290.00	23.56
334.05	59.01
352.20	16.82
368.05	5.75
396.15	93.22
440.10	100.00 – base peak

B. LC-MS/MS spectrum (ES-) of *N*,*N*'-di(2-hydroxybenzyl)ethylenediamine-*N*,*N*'-diacetic acid iron(III) sodium salt, Fe-HBED – base peak (m/z 440.10) fragmentation spectrum.



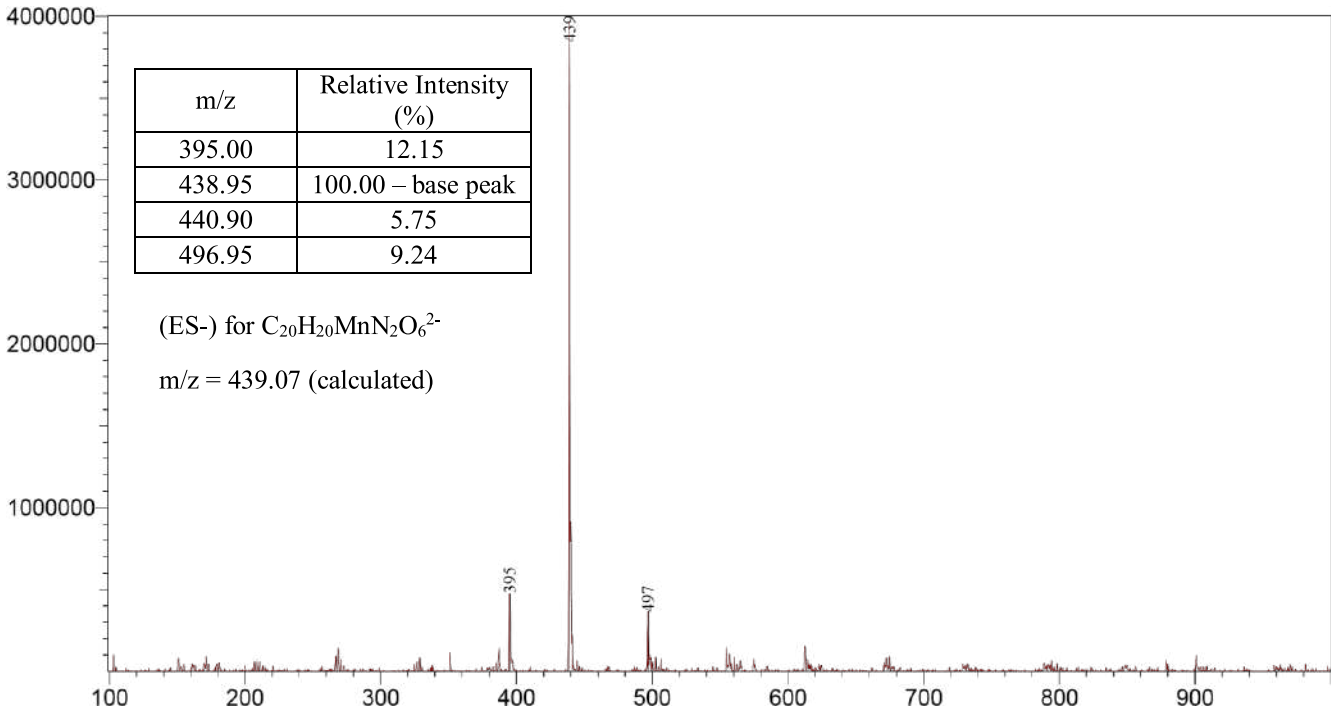
C. FT-IR spectrum of *N*,*N*'-di(2-hydroxybenzyl)ethylenediamine-*N*,*N*'-diacetic acid iron(III) sodium salt, Fe-HBED.



λ_{max1} = 274.0 nm; λ_{max2} = 309.0 nm; λ_{max3} = 491.5 nm;

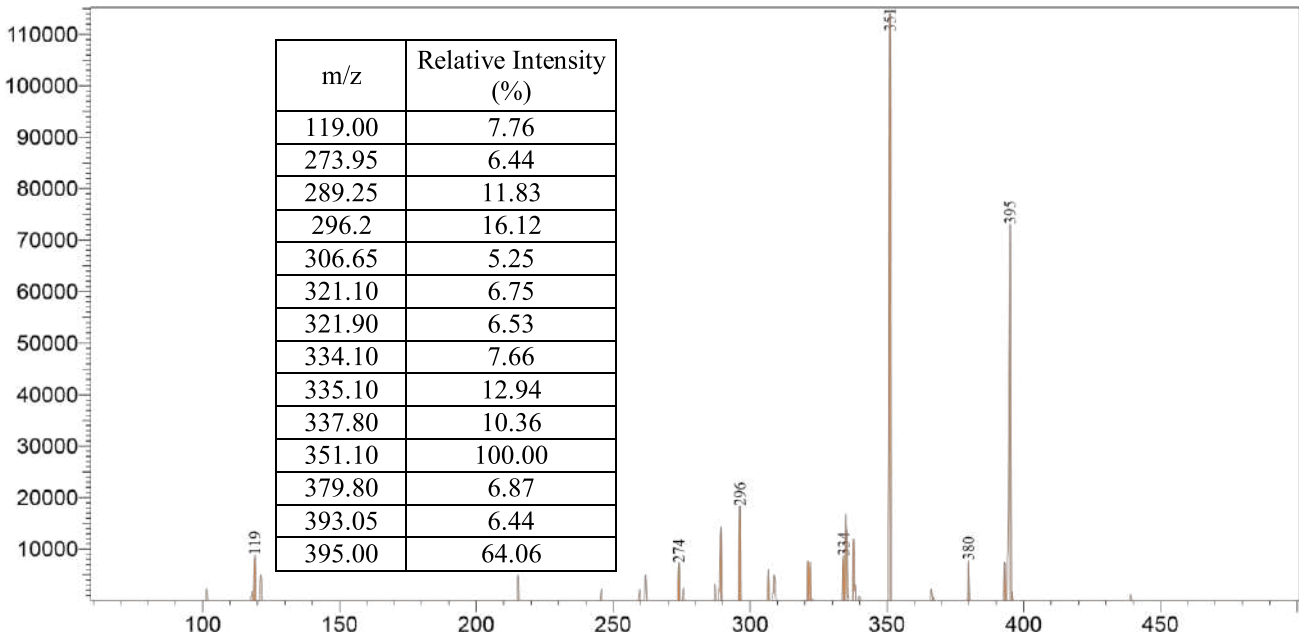
ε = 357.48 $dm^3 \cdot mol^{-1} \cdot cm^{-1}$

D. UV-VIS spectrum of *N*,*N*'-di(2-hydroxybenzyl)ethylenediamine-*N*,*N*'-diacetic acid iron(III) sodium salt, Fe-HBED.



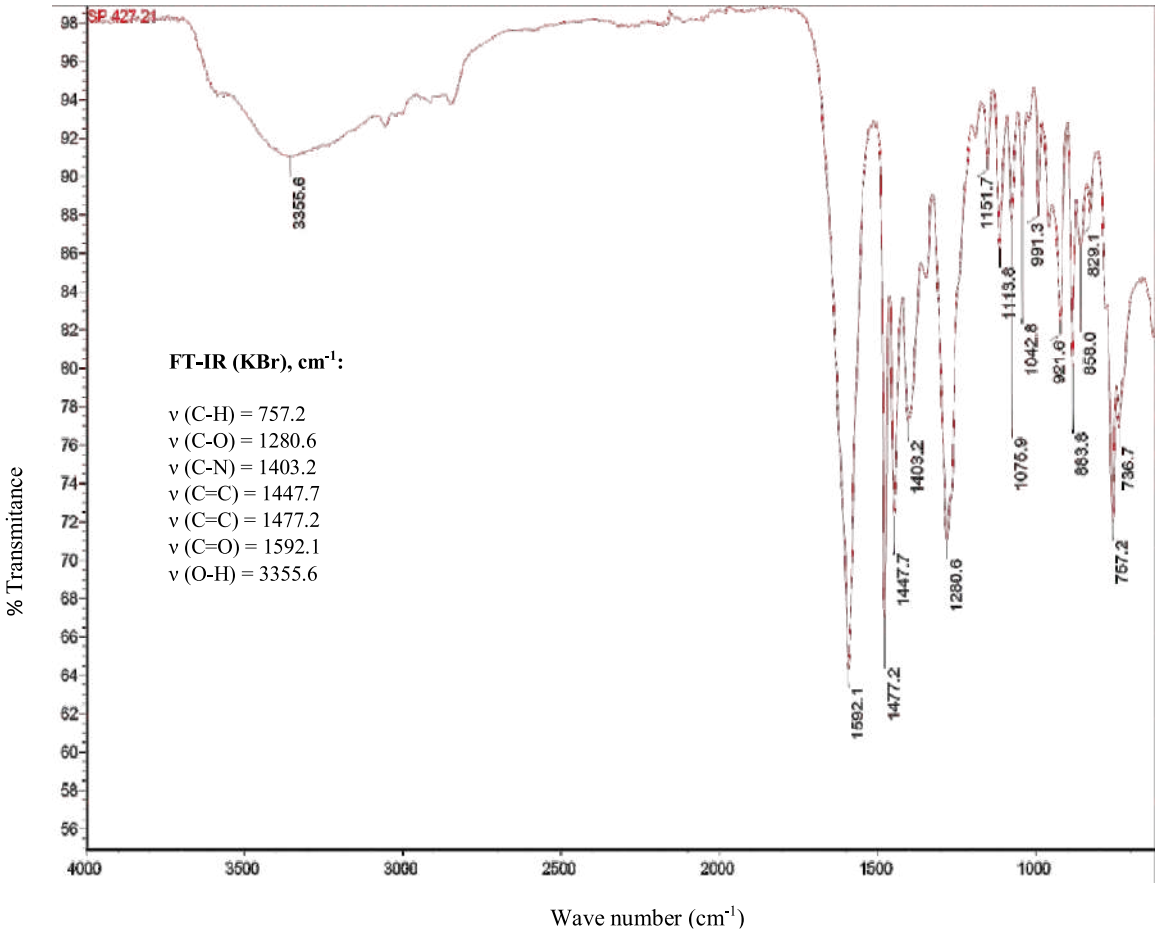
m/z	Relative Intensity (%)
395.00	12.15
438.95	100.00 – base peak
440.90	5.75
496.95	9.24

E. LC-MS/MS spectrum (ES-) of *N*,*N*'-di(2-hydroxybenzyl)ethylenediamine-*N*,*N*'-diacetic acid manganese(II) sodium salt, Mn-HBED.

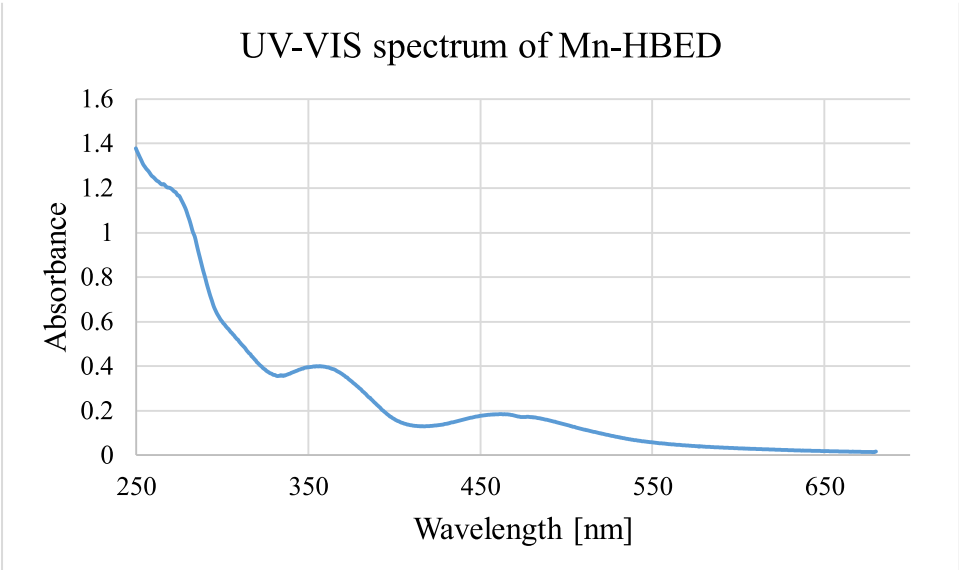


m/z	Relative Intensity (%)
119.00	7.76
273.95	6.44
289.25	11.83
296.2	16.12
306.65	5.25
321.10	6.75
321.90	6.53
334.10	7.66
335.10	12.94
337.80	10.36
351.10	100.00
379.80	6.87
393.05	6.44
395.00	64.06

F. LC-MS/MS spectrum (ES-) of *N*,*N*'-di(2-hydroxybenzyl)ethylenediamine-*N*,*N*'-diacetic acid manganese(II) sodium salt, Mn-HBED – base peak (m/z 438.95) fragmentation spectrum.



G. FT-IR spectrum of *N*,*N*'-di(2-hydroxybenzyl)ethylenediamine-*N*,*N*'-diacetic acid manganese(II) sodium salt, Mn-HBED.



λ_{max1} = 269.0 nm; λ_{max2} = 355.0 nm; λ_{max3} = 463.5 nm;

$\varepsilon = 675.01\ dm^3 \cdot mol^{-1} \cdot cm^{-1}$

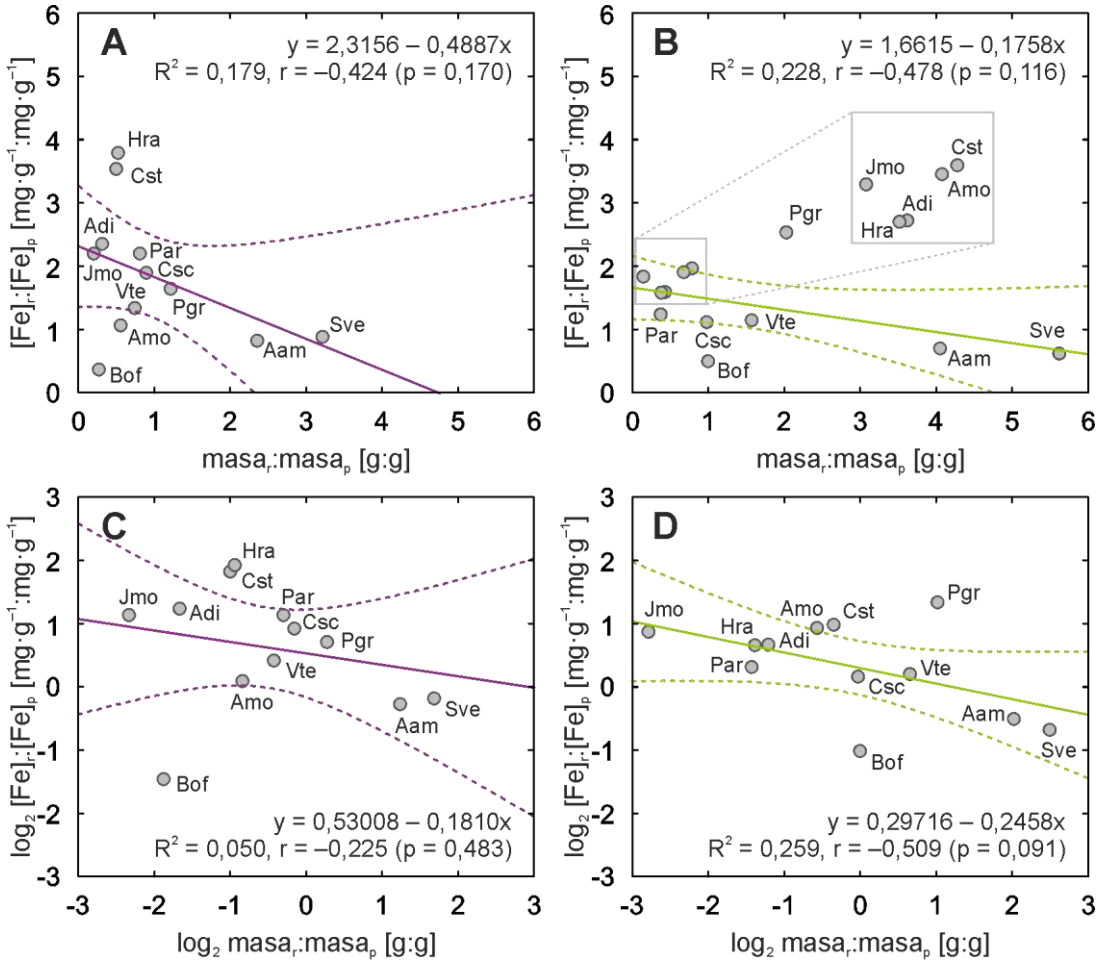
H. UV-VIS spectrum of *N*,*N*'-di(2-hydroxybenzyl)ethylenediamine-*N*,*N*'-diacetic acid manganese(II) sodium salt, Mn-HBED.

Supplementary Figure S1. LC-MS/MS spectra (A, B, E, F) , FTIR spectra (C, G) and UV-VIS spectra (D, H) of Fe-HBED and Mn-HBED.

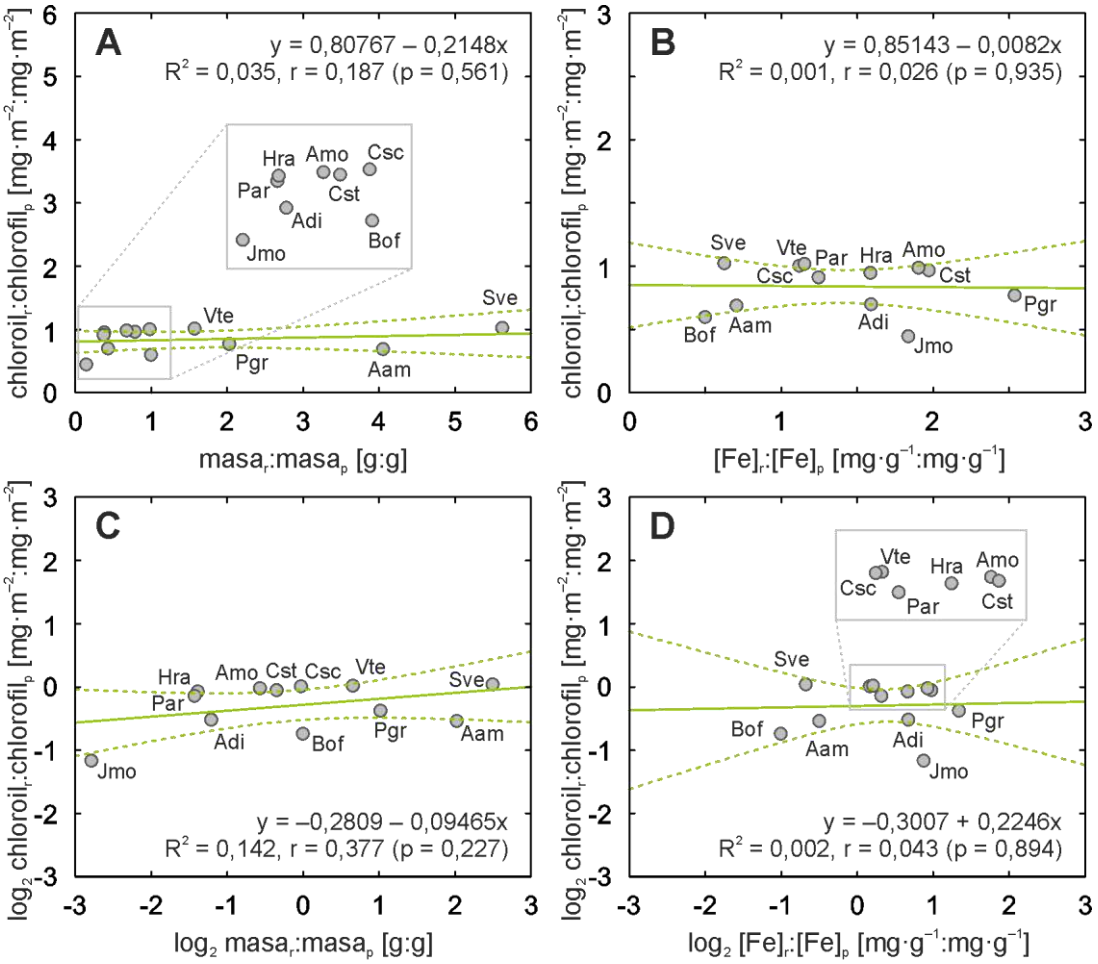
ZESTAWIENIE WYNIKÓW BADAŃ

Niepublikowany materiał uzupełniający

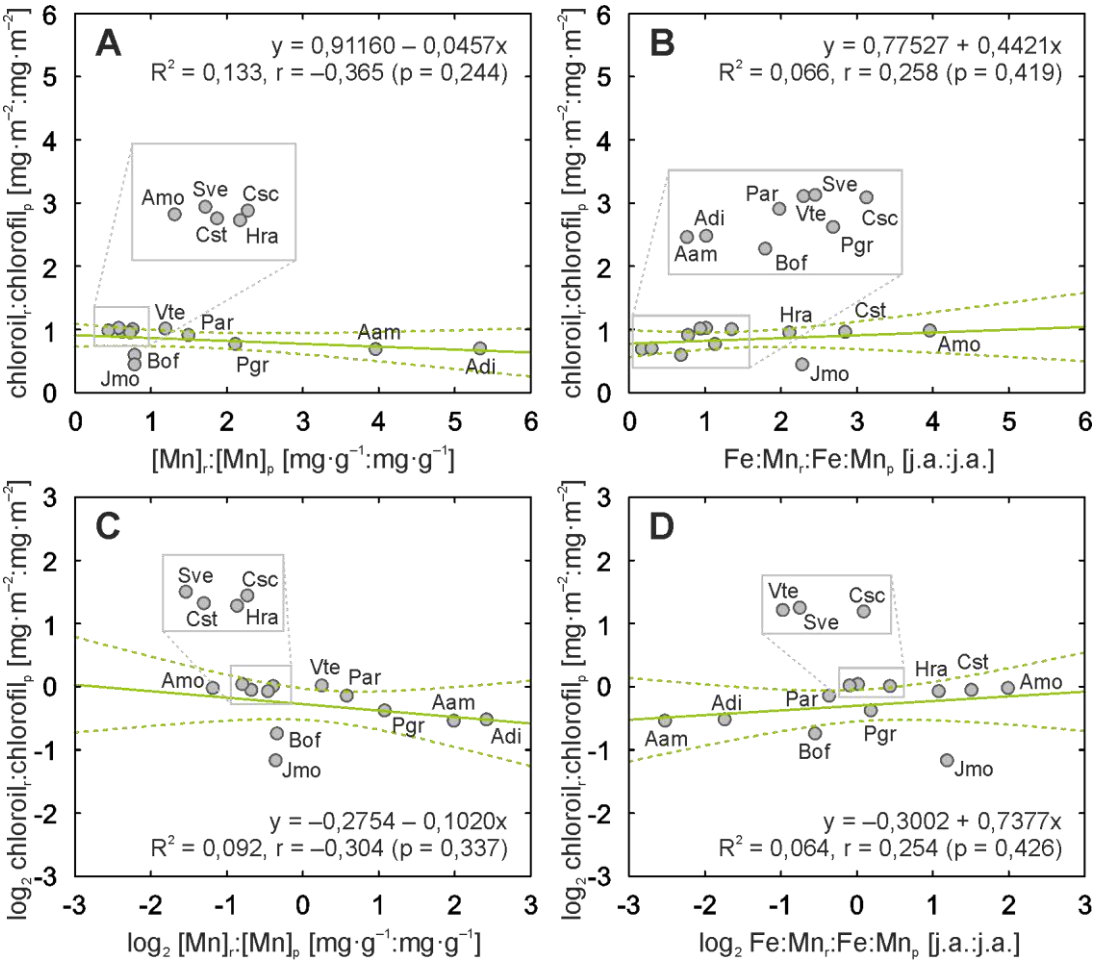
(Unpublished supplementary material)



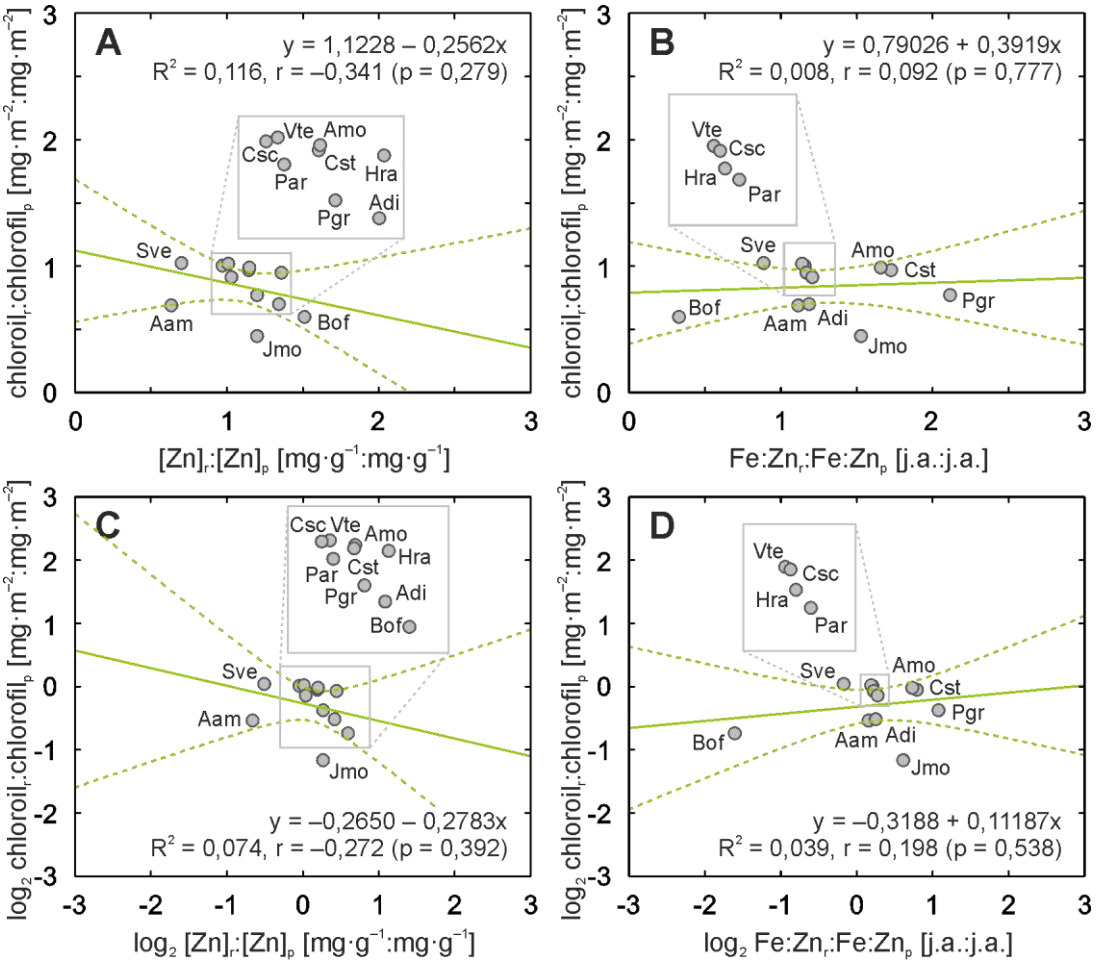
Rycina 2. Korelacja liniowa Pearsona między współczynnikiem kalcyfilowości, a współczynnikiem indukcji poboru żelaza przez alkalizację obliczona dla korzeni (A i C) oraz części nadziemnych (B i D) badanych gatunków. Współczynnik kalcyfilowości obliczono jako stosunek ilościowy między suchą masą danych organów roślin rosnących na glebie zasadowej (r) i na glebie kwaśnej (p) (na podstawie danych z prac P–1, P–2 i P–3), którego wartość X < 1 oznacza kalcyfobowość, a X > 1 kalcyfilowość; współczynnik indukcji poboru żelaza przez alkalizację obliczono jako stosunek ilościowy między zawartością żelaza w organie roślin rosnących na glebie zasadowej (r) i na glebie kwaśnej (p) (na podstawie danych z prac P–1, P–2 i P–3), którego wartość Y < 1 oznacza inhibicję poboru żelaza w warunkach alkalizacji, a Y > 1 zdolność do pobudzenia poboru żelaza w warunkach alkalizacji. Panele A i C przedstawiają analizę na danych nietransformoanych; panele B i D przedstawiają analizę na danych po transformacji (logarytm o podstawie 2). Barwne linie proste i przerywane przedstawiają, odpowiednio, równanie prostej oraz 95% przedział ufności. W trójliterowym skrócie nazwy gatunku pierwsza litera pochodzi od nazwy rodzajowej, a kolejne dwie od epitetu gatunkowego: Aam – *A. amellus*, Adi – *A. dioica*, Amo – *A. montanum*, Bof – *B. officinalis*, Csc – *C. scabiosa*, Cst – *C. stoebe*, Hra – *H. radicata*, Jmo – *J. montana*, Par – *P. arenaria*, Pgr – *P. grandiflora*, Sve – *S. verticillata*, Vte – *V. teucrium*.



Rycina 3. Korelacja liniowa Pearsona między współczynnikiem kalcyfilowości (A i C) oraz współczynnikiem indukcji poboru żelaza przez alkalizację (B i D), a współczynnikiem indukcji chlorozy przez alkalizację. Współczynnik kalcyfilności oraz współczynnik indukcji poboru żelaza przez alkalizację obliczono odpowiednio jako stosunek ilościowy między suchą masą części nadziemnych oraz jako stosunek ilościowy między zawartością żelaza w częściach nadziemnych roślin rosnących na glebie zasadowej (r) i na glebie kwaśnej (p) (na podstawie danych z prac P–1, P–2 i P–3), których wartość X < 1 oznacza odpowiednio kalcyfobowość lub inhibicję poboru żelaza, a X > 1 kalcyfilowość lub zdolność do pobudzenia poboru żelaza w warunkach alkalizacji. Współczynnik indukcji chlorozy przez alkalizację obliczono jako stosunek ilościowy między zawartością chlorofilu w liściach roślin rosnących na glebie zasadowej (r) i na glebie kwaśnej (p) (na podstawie danych z prac P–1, P–2 i P–3), którego wartość Y < 1 oznacza rozwój chlorozy na glebie zasadowej (rędzinie właściwej), a Y > 1 rozwój chlorozy na glebie kwaśnej (glebie bielicowej); Panele A i C przedstawiają analizę na danych nietransformoanych; panele B i D przedstawiają analizę na danych po transformacji (logarytm o podstawie 2). Barwne linie proste i przerywane przedstawiają, odpowiednio, równanie prostej oraz 95% przedział ufności. W trójliterowym skrócie nazwy gatunku pierwsza litera pochodzi od nazwy rodzajowej, a kolejne dwie od epitetu gatunkowego, zgodnie z opisem wcześniej opisem (Rycina 2).



Rycina 4. Korelacja liniowa Pearsona między współczynnikiem indukcji poboru manganu przez alkalizację (A i C) oraz współczynnikiem przesunięcia równowagi żelazowo-manganowej (B i D), a współczynnikiem indukcji chlorozy przez alkalizację. Współczynniki indukcji poboru manganu przez alkalizację oraz współczynnik przesunięcia równowagi żelazowo-manganowej obliczono odpowiednio jako stosunek ilościowy między zawartością manganu w częściach nadziemnych roślin oraz jako stosunek ilościowy między molowym współczynnikiem żelaza do manganu w częściach nadziemnych roślin rosnących na glebie zasadowej (r) i na glebie kwaśnej (p) (na podstawie danych z prac P–1, P–2 i P–3), których wartość X < 1 oznacza odpowiednio inhibicję poboru manganu lub przesunięcie równowagi w kierunku dominacji manganu nad żelazem w warunkach alkalizacji, a X > 1 zdolność do pobudzenia poboru manganu lub przesuniecie równowagi w kierunku dominacji żelaza nad manganem w warunkach alkalizacji. Współczynnik indukcji chlorozy przez alkalizację obliczono jako stosunek ilościowy między zawartością chlorofilu w liściach roślin rosnących na glebie zasadowej (r) i na glebie kwaśnej (p) (na podstawie danych z prac P–1, P–2 i P–3), którego wartość Y < 1 oznacza rozwój chlorozy na glebie zasadowej (rędzinie właściwej), a Y > 1 rozwój chlorozy na glebie kwaśnej (glebie bielicowej); Panele A i C przedstawiają analizę na danych nietransformoanych; panele B i D przedstawiają analizę na danych po transformacji (logarytm o podstawie 2). Barwne linie proste i przerywane przedstawiają, odpowiednio, równanie prostej oraz 95% przedział ufności. W trójliterowym skrócie nazwy gatunku pierwsza litera pochodzi od nazwy rodzajowej, a kolejne dwie od epitetu gatunkowego, zgodnie z opisem wcześniej opisem (Rycina 2). j.a. – jednostki arbitralne



Rycina 5. Korelacja liniowa Pearsona między współczynnikiem indukcji poboru cynku przez alkalizację (A i C) oraz współczynnikiem przesunięcia równowagi żelazowo-cynkowej (B i D), a współczynnikiem indukcji chlorozy przez alkalizację. Współczynniki indukcji poboru cynku przez alkalizację oraz współczynnik przesunięcia równowagi żelazowo-cynkowej obliczono odpowiednio jako stosunek ilościowy między zawartością cynku w częściach nadziemnych roślin oraz jako stosunek ilościowy między molowym współczynnikiem żelaza do cynku w częściach nadziemnych roślin rosnących na glebie zasadowej (r) i na glebie kwaśnej (p) (na podstawie danych z prac P–1, P–2 i P–3), których wartość X < 1 oznacza odpowiednio inhibicję poboru cynku lub przesunięcie równowagi w kierunku dominacji cynku nad żelazem w warunkach alkalizacji, a X > 1 zdolność do pobudzenia poboru cynku lub przesuniecie równowagi w kierunku dominacji żelaza nad cynkiem w warunkach alkalizacji. Współczynnik indukcji chlorozy przez alkalizację obliczono jako stosunek ilościowy między zawartością chlorofilu w liściach roślin rosnących na glebie zasadowej (r) i na glebie kwaśnej (p) (na podstawie danych z prac P–1, P–2 i P–3), którego wartość Y < 1 oznacza rozwój chlorozy na glebie zasadowej (rędzinie właściwej), a Y > 1 rozwój chlorozy na glebie kwaśnej (glebie bielicowej); Panele A i C przedstawiają analizę na danych nietransformoanych; panele B i D przedstawiają analizę na danych po transformacji (logarytm o podstawie 2). Barwne linie proste i przerywane przedstawiają, odpowiednio, równanie prostej oraz 95% przedział ufności. W trójliterowym skrócie nazwy gatunku pierwsza litera pochodzi od nazwy rodzajowej, a kolejne dwie od epitetu gatunkowego, zgodnie z opisem wcześniej opisem (Rycina 2). j.a. – jednostki arbitralne

10. Dyskusja

Choć wcześniejsze prace podejmowały problem żywienia żelazowego dziko występujących roślin wyższych (w tym murawowych) i potwierdzały istnienie różnic w wymaganiach względem tegoż pierwiastka (Clymo, 1962; Misra i Tyler, 1999; Wang i wsp., 2022), prace P–1, P–2 i P–3 stanowią pierwszą próbę ujęcia problemu etiologii chlorozy zależnej od żelaza przez pryzmat przeciwstawności badanych typów muraw i powszechności zależności przyczynowo-skutkowych, wpisując się w sygnalizowaną potrzebę prowadzenia badań porównawczych (Bothe, 2015).

Podsumowując prace P–1, P–2 i P–3 należy wskazać przede wszystkim brak istotnego powiązania między wymaganiami względem odczynu gleby, a wymaganiami żywieniowymi względem żelaza (Rycina 2). Na podstawie uzyskanych danych należy zatem odrzucić pierwszą ogólną hipotezę badawczą (HO–1). Fakt ten uniemożliwia również stworzenie w oparciu o zaprezentowane dane modelu pozwalającego na przewidywanie procesu poboru żelaza przez rośliny o określonych preferencjach względem typu gleby i *vice versa*. Należy zatem przyjąć hipotezę alternatywną, mówiącą, że wymagania względem odczynu gleby oraz względem dostępności żelaza są rozdzielne. Biorąc jednak pod uwagę całokształt wyników (Rycina 2), nie jest wykluczone, że stworzenie modelu łączącego oba wymagania będzie w przyszłości możliwe przy rozszerzeniu zakresu badań lub uszczegółowieniu kryteriów wyboru gatunków. Hipotetyzując, model taki prawdopodobnie wskazywałby, że istnieje przeciętna korelacja ujemna między współczynnikiem kalcyfilowości, a współczynnikiem indukcji poboru żelaza przez alkalizację. W ujęciu funkcjonalnym oznaczałoby to, że kalcyfoby mają wysokie wymagania względem żelaza i posiadają adaptacje pozwalające na indukowane zwiększenie poboru tegoż pierwiastka w warunkach częściowej alkalizacji podłoża (cechy organizmu w większym stopniu kontrolują proces niż czynniki środowiska), a kalcyfile mają niskie wymagania względem żelaza i pobierają tenże pierwiastek zgodnie z gradientem czynników edaficznych (czynniki środowiska w większym stopniu kontrolują proces niż cechy organizmu).

Interpretując informacje odnoszące się do wymagań badanych roślin warto zwrócić uwagę przede wszystkim na skalę zróżnicowania ich reakcji na badane czynniki. *B. officinalis* jest gatunkiem o raczej szerokim zakresie preferencji substratowej, który jako jedyny (w przeciwieństwie do *C. scabiosa*, *P. arenaria* i *V. teucrium*) wykazuje chlorozę powodowaną niedoborem żelaza bez kluczowych cech „paradoksu chlorozy zależnej od żelaza". Dla odróżnienia, jeden gatunek kalcyfilowy (*P. grandiflora*) oraz dwa gatunki kalcyfobowe (*A. dioica* i *J. montana*) przejawiały objawy niedoboru żelaza pasujące do kanonicznego opisu „paradoksu". *A. montanum*, *C. stoebe* i *H. radicata* są natomiast doskonałymi przykładami gatunków kalcyfobowych posiadających adaptacje umożliwiające nasilony pobór żelaza z gleby zasadowej, przy czym nie wykazują one jakichkolwiek niedoborów tegoż pierwiastka. Co ciekawe, wśród wybitnych kalcyfilów można odnaleźć zarówno rośliny odporne (*S. verticillata*), jak i podatne na rozwój niedoborów żelaza (*A. amellus*), przy czym ograniczenia te nie noszą znamion „paradoksu". Zróżnicowanie wymagań względem dostępności żelaza w zależności od preferencji względem odczynu gleby była wielokrotnie sygnalizowana w przeszłości,

jednakże dyskutowana była wyłącznie w kontekście występowania w obrębie danego typu muraw (Hutchinson, 1967) lub liczb wskaźnikowych Ellenberga (Bartelheimer i Poschlod, 2016), bez potwierdzenia rzeczywistej preferencji substratowej badanych gatunków. Ponadto, niniejsza praca jako pierwsza poddaje w wątpliwość istnienie ścisłego związku między skrajną lub stenotopową preferencją substratową gatunków murawowych względem czynników edaficznych, a ich odpornością na rozwój silnych niedoborów żelaza. Analizując uzyskane wyniki można zaproponować, że nie wszystkie kalcyfile muszą posiadać adaptacje warunkujące indywidualne zdolności do pozyskiwania żelaza z form nierozpuszczalnych (Bothe, 2015).

Problem chlorozy dotknął przeszło 40% ogółu badanych gatunków (5 z 12), jednakże w podobnym stopniu dotyczył on zarówno gatunków uznawanych za zasadolubne (*A. amellus*, *B. officinalis* i *P. grandiflora*), jak i tych uznawanych za kwasolubne (*A. dioica* i *J. montana*). Zróżnicowanie dotyczyło również dawki chelatu przełamującej niedobór żelaza na glebie zasadowej (5 µmol · kg^{-1} gleby dla *A. amellus*, *P. grandiflora*, *A. dioica* i *J. montana* oraz 25 µmol · kg^{-1} gleby dla *B. officinalis*) oraz sposobu w jakim dochodziło do wyrównania chlorozy (niepełne w stosunku do wartości obserwowanej gdy rośliny rosły na glebie kwaśnej dla *A. dioica* i *J. montana* i pełne dla pozostałych gatunków). Na podstawie niniejszych badań nie można zatem stwierdzić jednoznacznie, że problem chlorozy dotyczy wyłącznie gatunków kwasolubnych, tj. niedopasowanych do zasadowego odczynu podłoża. Potwierdza to zbiorcza analiza korelacji wykonana w oparciu o dane zaprezentowane w pracach P–1, P–2 oraz P–3 (Rycina 3). Istnieją przez to istotne przesłanki do odrzucenia drugiej ogólnej hipotezy badawczej (HO–2). Oznacza to, że występowanie chlorozy zależnej od żelaza nie zależy ani od preferencji badanych gatunków względem typu gleby, ani od ich zdolności do pobierania żelaza z gleby zasadowej. Choć dane potwierdzają, że chloroza zależna od odczynu gleby jest objawem występującym u obu grup gatunków, to stoją one w jawnej sprzeczności ze stwierdzeniem, że niedobór żelaza (w ujęciu prostym, tj. jednoczynnikowym) jest główną przyczyną powstawania chloroz (Hutchinson, 1967). Oznacza to istnienie innej, współwystępującej ze zmianami dostępności żelaza przyczyny lub istnienie przyczyny złożonej (w której dostępność żelaza jest jednym z kilku komponentów).

Reorientacja poszukiwań przyczyn badanych chloroz z roli żelaza na inne pierwiastki zmusiła do postawienia pytania o antagonizm między żelazem, a makro- i mikroskładnikami. Sugerując się informacjami o roli manganu (Foy, 1984; Horst, 1988), cynku (Kaur i Garg, 2021) oraz antagonizmu tychże pierwiastków z żelazem w procesie rozwoju chlorozy (Somers i Shive, 1942; Rai i wsp., 2021), obliczono molowe stosunki ilościowe tychże pierwiastków (prace P–1, P–2 i P–3). Uzyskane dane wskazały, że najbardziej prawdopodobną przyczyną niedoborów żelaza oraz rozwoju chlorozy jest antagonizm żelaza z manganem (dla gatunków z kserotermicznych muraw wapieniolubnych: *A. amellus*, *B. officinalis* i *P. grandiflora*) lub antagonizm z manganem i cynkiem jednocześnie (dla gatunków z piaszczystych muraw bezwapiennych: *A. dioica* i *J. montana*). Oznacza to, że etiologia chloroz zależnych od żelaza ma charakter złożony. Jest to jednocześnie pierwsze doniesienie o powszechności złożonej etiologii chlorozy, której istnienie proponowane było wcześniej wyłącznie w oparciu o dane odnoszące się

do stosunkowo wąskiej grupy gatunków modelowych (*Arabidopsis thaliana* L.) lub uprawnych (*Hordeum vulgare* L., *Phaseolus vulgaris* L., *Vigna unguiculata* (L.) Walp. I *Zea mays* L.; Therby-Vale i wsp., 2022).

Przeprowadzono również analizę zbiorczą wyników prac P–1, P–2 i P–3, która wskazała, że zawartość manganu i cynku, jak również stosunek ilościowy żelaza do manganu i cynku w częściach nadziemnych nie koreluje istotnie z współczynnikiem indukcji chlorozy przez alkalizację (Rycina 4; Rycina 5). Należy jednak podkreslić, że analizę zaburzał silnie odstający przypadek *J. montana*, u którego rozwój chlorozy może mieć znacznie bardziej złożony charakter (prawdopodobnie wynika z kombinacji zaburzeń związanych z poborem i transportem wszystkich oznaczonych pierwiastków). Choć nie ma przesłanek do wykluczenia *J. montana* z modelu, to po eliminacji tegoż gatunku z analizy, model oparty o stosunek ilościowy żelaza do manganu (lecz nie o stosunek ilościowy żelaza do cynku) wskazywałby silną ($r = 0,669$), istotną ($p = 0,024$) korelację tegoż czynnika z współczynnikiem indukcji chlorozy; jednakże w oparciu o taki model tylko niespełna połowę przypadków chloroz można by tłumaczyć wprost proporcjonalną zmianą stosunków ilościowych między żelazem i manganem ($R^2 = 0,448$). Brak współwystępowania zaburzeń w biosyntezie i/lub utrzymaniu chlorofilu i preferencji substratowych lub wzorców poboru makro- i mikroelementów w ujęciu całościowym wskazuje, że etiologia chloroz zależnych od żelaza ma charakter zróżnicowany (specyficzny dla gatunku). Nie można zatem ująć problemu niedoboru żelaza w sposób generalny, co podkreśla rolę badań szczegółowych nad ekologią gatunków w zrozumieniu ekologii zespołów wielogatunkowych i jest spójne z aktualnym podejściem badawczym (Shinohara i wsp., 2023).

Podsumowując problem etiologii chloroz zależnych od żelaza należy wskazać, że rozwój tego objawu u gatunków podatnych na niedobory żelaza uwarunkowany jest antagonizmem między żelazem i manganem oraz cynkiem. Powyższa interakcja powodowana jest nadmiarowym (patofizjologicznym) poborem manganu i cynku (przez transportery o niskiej specyfice względem wymienionych pierwiastków) oraz ich alokacją do części nadziemnej, gdzie wywierają działanie toksyczne. Choć ostatnie doniesienia silnie sugerowały istnienie takiej zależności (Therby-Vale i wsp., 2022), prace P–1, P–2 oraz P–3 po raz pierwszy ujmują problem w sposób całościowy. Co ważne, wyżej proponowany mechanizm, ze względu na wybór badanych gatunków oraz rodzaj prowadzonych badań, daje możliwość interpretacji zjawisk w odniesieniu do procesów zachodzących w przyrodzie.

Rozwój chlorozy u gatunków kalcyfilowych sugeruje również istnienie ciekawej implikacji o wzajemnej zależności gatunków kserotermicznych muraw nawapiennych lub ich zależności od innych grup organizmów w kontekście żywienia żelazowego. Znany jest pozytywny wpływ traw (produkujących znacznie ilości fitosideroforów) na podatne względem chlorozy zależnej od żelaza rośliny niebędące trawami (Dai i wsp., 2019; Ueno i wsp., 2021). Biorąc pod uwagę, że *A. amellus*, *B. officinalis* i *P. grandiflora* zazwyczaj wzrastają w obrębie muraw bez objawów chlorozy, należy uznać za prawdopodobne, że czerpią one korzyści z uwalnianej przez inne organizmy puli dostępnego żelaza. Wpisuje

się to w przedstawiany ostatnio, złożony model wykraczający poza ramy ekologii poszczególnych gatunków i ich indywidualnych wymagań, a nawet poziomu holobiontów (Vélez-Bermúdez i Schmidt, 2022). Zatem, ujęcie puli dostępnego w glebie żelaza jako współdzielonego zasobu w postaci tzw. „dobra wspólnego" oznaczałoby, że poza konkurencją o zasób, dochodzi również do komensalizmu (między gatunkami podatnymi na chlorozę, a gatunkami zwiększającymi pulę dostępnego żelaza ponad swoje potrzeby; Dai i wsp., 2019), ale i allelopatii niebezpośredniej (Zeng 2014; między gatunkami wrażliwymi na toksyczność zależną od żelaza, a gatunkami zwiększającymi pulę dostępnego żelaza ponad swoje potrzeby). Biorąc jednak pod uwagę udział innych organizmów w kształtowaniu dostępności tegoż pierwiastka w glebie (Dakora i Phillips, 2002; Hider i Kong, 2010), pełne zrozumienie zjawiska chlorozy zależnej od żelaza w skali ekosystemów oraz w ujęciu czasoprzestrzennej zmienności będzie wymagało znacznego nakładu badań.

Ograniczona dostępność makro- i mikroelementów stanowi grupę stresorów bardzo powszechnie oddziałujących na rośliny (Schulze i wsp., 2019). Rozwijający się stres powoduje dysfunkcje na wielu poziomach organizacji organizmu, a pogłębiające się załamanie metabolizmu pierwotnego i specjalistycznego (wtórnego) wpływa negatywnie na wzrost i rozwój (Scheres i van der Putten, 2017; Schulze i wsp., 2019). Wyniki prac P–1, P–2 i P–3 są zgodne z powyższymi stwierdzeniami – stres powodowany niedoborem żelaza ma daleko idące skutki, a zahamowanie wzrostu może być związane z intensyfikacją działania mechanizmów obronnych (Scheres i van der Putten, 2017). W konsekwencji, może dochodzić do zmniejszenia przeżywalności osobników, również w wyniku konkurencji bezpośredniej o przestrzeń z sąsiadującymi osobnikami, a w efekcie o dostęp do światła (Mortimer, 1992). Jest to wysoce prawdopodobne, gdyż rozmiar liści osobników poddanych stresowi był często mniejszy niż rozmiar tychże organów u osobników rosnących w warunkach optimum.

Ponadto, funkcjonalność liści przejawiających objawy chlorozy była również ograniczona, choć rozpatrując wyniki analiz fluorescencji chlorofilu *a* można się dopatrzyć pewnych mechanizmów zabezpieczających badane gatunki na okoliczność przejściowych zaburzeń w gospodarce żelazem (umożliwiające przeżycie zanim dojdzie do kompensacji poniesionych strat). Jednym z mechanizmów radzenia sobie ze stresem powodowanym nadmiarem promieniowania fotosyntetycznie czynnego jest jego odprowadzenie, zarówno na drodze promienistej, jak i niepromienistej, m.in., poprzez oddanie ciepła, fluorescencji lub transportu energii do sąsiadujących układów (Kalaji, 2011). W tym kontekście, zwiększone wartości DI_0/RC, świadczące o nasilonym rozpraszaniu energii wzbudzenia, obserwowane było u wszystkich gatunków wykazujących objawy niedoboru żelaza. Jednocześnie, notowano spowolnienie transportu elektronów (ET_0/RC) w fotoukładach. Podobną reakcję wykazywały inne gatunki roślin wyższych poddanych eksperymentalnym zaburzeniom w żywieniu żelazowym (Luna i wsp., 2020; Prity i wsp., 2020). Wynika z tego, że wszystkie badane gatunki podatne na rozwój chlorozy posiadają adaptacje pozwalające na (przynajmniej częściowe) zredukowanie szkodliwego działania nadmiarowego promieniowania fotosyntetycznie czynnego poprzez oddanie części energii, co może ograniczyć skalę stresu oksydacyjnego i fotouszkodzeń (Hall i Rao, 1999; Allahverdiyeva i Aro, 2012). Niestety, mimo że wzorzec reakcji wszystkich gatunków chlorotycznych

na niedobór żelaza był bardzo zbliżony, powyższe parametry nie mogą stanowić specyficznego wskaźnika (markera) odpowiedzi roślin na niedobory żelaza. Zwiększenie wartości DI_0/RC oraz zmniejszeni wartości PI_{ABS} oraz (niekiedy) F_V/F_M jest wysoce niespecyficznym, typowym skutkiem zaburzonego funkcjonowania fotoukładów w wyniku rozwoju chlorozy, niezależnie od jej etologii (Kalaji, 2011). Oznacza to, że zarówno niedobory magnezu, wapnia, azotu i żelaza, jak i toksyczność manganu dają te same objawy (utrata chlorofilu) i powodują takie same zmiany w fluorescencji chlorofilu *a* (Kalaji, 2011; Liu i wsp., 2021). Zatem, w kontekście praktycznej diagnostyki niedoborów mineralnych, symptomatologia nadal pozostaje podstawowym narzędziem przydatnym do stwierdzenia etiologii obserwowanych niedoborów.

Powyższe wyniki były zgodne z danymi odnoszącymi się do pozostałych reakcji stresowych, związanych z funkcjonowaniem enzymatycznego aparatu antyoksydacyjnego (u gatunków zasadolubnych) oraz metabolizmem specjalistycznym (u gatunków kwasolubnych). Silny stres związany był z rozwojem odpowiedzi noszących znamiona patofizjologii, jednakże ich wzorzec był specyficzny dla gatunku (prace P–1 i P–2) Nieadekwatna reakcja obronna uniemożliwia kompensację poniesionych strat i prowadzi w efekcie do zaburzenia homeostazy organizmu (Kochhar i Gujral, 2020), co jest związane z osłabieniem poboru składników odżywczych (Erb i Kliebenstein, 2020). To z kolei prowadzi często do przesunięcia równowagi oksydo-redukcyjnej organizmu i pociąga za sobą rozwój stresu oksydacyjnego (Bartosz, 1997). W kontekście niniejszych badań, zaburzenia związane z żywieniem mineralnym upośledzają zdolności roślin do przeciwdziałania stresowi oksydacyjnemu co może pogarszać funkcjonowanie zaburzonego (na poziomie fotoukładów) aparatu fotosyntetycznego, w którym dochodzi do ciągłego wycieku elektronów (Bartosz, 1997; Andressen i wsp., 2018).

Na kanwie wyników odnoszących się do reakcji stresowych badanych roślin, należy uznać, że nie ma przesłanek do odrzucenia trzeciej hipotezy ogólnej (HO–3). Oznacza to, że zarówno gatunki z piaszczystych muraw bezwapiennych, jak i z kserotermicznych muraw wapieniolubnych doświadczają silnego stresu powodowanego skrajnym niedopasowaniem substratowym i nieadekwatną do ich potrzeb dostępnością żelaza. Choć zmiany w funkcjonowaniu aparatu fotosyntetycznego w obliczu stresu powodowanego żelazem wydają się mieć jednorodny charakter, to badane gatunki w różny sposób reagują na poziomie metabolizmu specjalistycznego (kalcyfoby) i enzymatycznego systemu antyoksydacyjnego (kalcyfile), co sygnalizuje istnienie indywidualnych progów tolerancji dla badanych czynników edaficznych.

Wpływ odczynu (Baskin i Baskin, 2014) oraz dostępności glinu (Abedi i wsp., 2013) na kiełkowanie nasion wydaje się być dobrze poznany; uważa się, że skrajne wartości pH (silnie kwaśne lub silnie zasadowe) oraz wysoka dostępność jonów glinu hamują ten proces. Zazwyczaj dane te nie odnoszą się jednak do gatunków z siedlisk, gdzie skrajne wartości obu czynników mogą powodować głęboką specjację. Co prawda w literaturze można odnaleźć informacje, że zarówno gatunki preferujące podłoże skrajnie kwaśne lub zasadowe wykazują adaptacje strategii kiełkowania charakterystyczne dla grupy, ale badania te dotyczą muraw wysokogórskich (Tudela-Isanta i wsp., 2018). Przytoczone

wyniki pokazały , że nasiona gatunków kalcyfilnych kiełkują tak samo dobrze w przedziale odczynu 4,5–7,5 lub (rzadko) zdolność nasion do wykiełkowania maleje wraz ze wzrostem odczynu, podczas gdy nasiona roślin kalcyfobowych kiełkują tak samo dobrze w szerokim zakresie wartości pH (4,5–7,5) lub (rzadko) ich zdolność do wykiełkowania maleje wraz ze wzrostem odczynu (Tudela-Isanta i wsp., 2018). Praca P–4 nie potwierdza tejże zależności dla gatunków niealpejskich, występujących w obrębie ciepłych i suchych muraw, gdyż obserwowana różnorodność wymagań względem odczynu w obrębie obu typów muraw była znaczna. Sugeruje to, że mikromozaikowatość siedlisk obu typów muraw (Leuchner i Ellenberg, 2017) może wpływać pozytywnie na koegzystencję gatunków, których nisze kiełkowania są różne. Choć uważa się, że rośliny kwasolubne cechują się wyższą tolerancją względem glinu niż gatunki zasadolubne, to stwierdzenie to odnosi się do bardzo wczesnych etapów wzrostu siewki oraz dalszych etapów rozwoju (Abedi i wsp., 2013). Nie jest to jednak spójne z badaniami nad procesem kiełkowania (Abedi i wsp., 2013), gdzie jasno wskazano, że zdolność nasion *Trifolium arvense* L. (EIV R = 2) do zakończenia kiełkowania jest hamowana przez stężenie glinu równe lub wyższe 2 mmol · dm^{-3}, podczas gdy procent kiełkowania *J. montana* (EIV R = 3), *Helichrysum arenarium* (L.) Moench (EIV R = 5) i *Erigeron* acris L. (EIV R = 8) nie zależał od obecności glinu, aż do stężenia równego 10 mmol · dm^{-3}. W tym aspekcie praca P–4 jest spójna z poprzednimi wynikami badań, ale poprzez zakres testowanych hipotez wskazuje, jako pierwsza, że szacunki odnoszące się do preferencji względem odczynu gleby nie są tożsame z tolerancją na obecność wolnego glinu, gdy brane są pod uwagę strategie kiełkowania.

Co zaskakujące, choć żelazo i mangan są bardzo powszechnie występującymi w glebie pierwiastkami, pełniącymi rolę mikroelementów w życiu roślin oraz należącymi do grupy metali ciężkich, ich rola w procesie kiełkowania nie była szczegółowo badana. Stąd też brak jest usystematyzowanej wiedzy odnośnie wpływu (lub braku wpływu) obu pierwiastków. Problem ten zdają się pomijać zarówno prace z zakresu biologii, jak i nauk o środowisku (Nagajyoti i wsp., 2010; Kranner i Colville, 2011). Warto wspomnieć, że wpływ obu pierwiastków był badany do tej pory w sposób jednostkowy, najczęściej w kontekście roślin uprawnych, o dużym znaczeniu użytkowym dla człowieka jak pszenica zwyczajna (*Triticum aestivum* L.; Reis i wsp., 2018;) i ryż siewny (*Oryza sativa* L.; Wang i wsp., 2020). Prace te jednak skupiały się na zakresie stężeń będących przedmiotem rozważań toksykologii lub (ewentualnie) badane stężenia były charakterystyczne dla gleb wysoce eutroficznych lub wilgotnych i/lub stale lub okresowo zalewanych, gdzie dostępność żelaza i manganu może być bardzo wysoka, a trwały lub przejściowy rozwój hipoksji w glebie faworyzuje przesunięcie specjacji tychże pierwiastków w stronę toksycznych form zredukowanych (Strawn i wsp., 2020). Ogranicza to możliwość porównania danych w kontekście gleb, w których żelazo i mangan występują w formie kompleksów, a jeśli pierwiastki te występują w postaci jonowej, to w znacznie mniejszej ilości i formie utlenionej (Winkelmann, 2007; Bothe, 2015; Strawn i wsp., 2020). W powyższym kontekście, praca P–4 jako pierwsza wskazuje, że żelazo i mangan (w formie i ilości zbliżonej do wartości notowanych w danych siedliskach) odgrywają rolę inhibitora w regulacji procesu kiełkowania. Rola ta ma jednak charakter drugorzędny.

Gatunkowa specyfika wielkości nasion współwarunkuje zdolność gatunków do kolonizacji luk w fitocenozie, tworzenia glebowych banków nasion oraz fenologię wschodów; uważa się przy tym, że nasiona roślin wielkonasiennych lepiej radzą sobie z suboptymalnym natężeniem fizycznych czynników środowiska (temperatura, oświetlenie, głębokość zalegania nasion), a proces ich kiełkowania jest mniej uzależniony od tychże warunków niż kiełkowanie roślin drobnonasiennych (Silvertown, 1981). Wyniki pracy P–4 nie potwierdzają tej zależności dla badanych czynników chemicznych (lub według alternatywnej interpretacji, gdy nasiona kiełkują w warunkach supraoptymalnego natężenia danego czynnika). Oznacza to, że hipotetyczne założenie o istnieniu związanej z wielkością nasion bariery dla udanej kolonizacji miejsc (Silvertown, 1981) wydaje się nie mieć potwierdzenia w badaniach nad stresorami chemicznymi. Dobitnym tego przykładem jest porównanie gatunków o najcięższych diasporach (*C. stoebe*, *C. scabiosa* i *E. vulgare*), w którym to gatunek o najniższej masie diaspor (*C. stoebe*) wykazywał najszerszą tolerancję ekologiczną. Co więcej, rośliny drobnonasienne, których diaspory mają masę ok. 7–11-krotnie mniejszą niż *C. stoebe*, miały porównywalnie szeroki (*D. carthusianorum* i *R. acetosella*) lub zdecydowanie węższy (*P. media* i *V. teucrium*) zakres tolerancji. Oznacza to, że sama masa diaspor nie determinuje ich zdolności do wykiełkowania w warunkach ciągłej ekspozycji na dany czynnik chemiczny, co sugeruje, że zdolność ta może być bardziej związana z strukturalno-funkcjonalnymi adaptacjami celowanymi przeciwko danemu stresorowi. Jest to zatem kolejny element różnicujący ekologię badanych gatunków na wczesnym etapie ontogenezy.

Ujmując ogół wyników przedstawionych w ostatnim etapie prac, należy odrzucić czwartą ogólną hipotezę roboczą (HO–4) zakładającą istnienie specyficznych dla danej grupy gatunków przystosowań polegających na ścisłym dopasowaniu ich strategii kiełkowania do ikonicznych cech ich siedlisk (w tym przypadku dostępności żelaza, manganu i glinu oraz odczynu). Należy przez to przyjąć alternatywną hipotezę o specyficznych dla gatunku wymaganiach pozwalających na zakończenie procesu kiełkowania, a więc tej części niszy ekologicznej, która odnosi się do strategii kiełkowania. Sugeruje to, że zróżnicowane strategie kiełkowania mogą się przyczyniać do ograniczenia konkurencji badanych gatunków, pozwalając na ich współwystępowanie (koegzystencję) w obrębie wysoce heterogenicznych (mikromozaikowatych) siedlisk.

Podsumowując, niniejsza praca wskazała daleko posunięte zróżnicowanie wymagań roślin występujących naturalnie w obrębie obu typów muraw. Wykazany niski stopień wzajemnego powiązania wymagań względem odczynu gleby i względem dostępności żelaza, różnorodność stopnia kalcyfilności i kalcyfobowości badanych gatunków oraz złożoność etiologii niedoborów żelaza pozwalają na lepsze zrozumienie zjawiska „paradoksu chlorozy zależnej od żelaza", które może dotykać rośliny z piaszczystych muraw bezwapiennych i kserotermicznych muraw wapieniolubnych. Kwestią otwartą pozostają badania nad ekologią pozostałych roślin wyższych (przede wszystkim traw) oraz roślin jednorocznych, występujących na obu typach siedlisk. Oznacza to, że gatunkowa specyfika reakcji roślin na różnych etapach ontogenezy może stanowić bogaty obszar dla dalszych badań.

11. Wnioski

W świetle wyników badań oraz w kontekście postawionych celów i hipotez ogólnych upoważnione staje się postawienie następujących wniosków odnoszących się bezpośrednio do badanych w niniejszej pracy gatunków:

- **Preferencje względem typu gleb i dostępności żelaza są rozdzielne.** Choć większość gatunków wykazuje preferencję substratową zgodną z oczekiwaniami (gatunki kwasolubne występujące w obrębie suchych muraw bezwapiennych rosną lepiej na glebie kwaśnej, a zasadolubne z kserotermicznych muraw wapieniolubnych na glebie zasadowej), to część gatunków wykazuje preferencje o znamionach eurytopowości. Gatunki należące do tej samej rodziny (Asteraceae i Lamiaceae), wykazują zróżnicowane wzorce reakcji na typ podłoża oraz dostępność żelaza. Wśród badanych roślin odnaleźć można zarówno gatunki podatne, jak i odporne na rozwój niedoborów żelaza.

- **Chloroza zależna od żelaza ma charakter zróżnicowany i złożony.** Niedobory żelaza objawiają się rozwojem chlorozy, która to znoszona jest w wyniku zwiększenia dostępności żelaza w glebie poprzez podanie go w formie łatwo przyswajalnej (chelatu). Dawka niezbędna do zniesienia objawów jest specyficzna dla gatunku. Chloroza nie zawsze wynika z deficytu żelaza *per se* i nie jest skutkiem zaburzeń w jego translokacji. Chloroza związana z alkalizacją podłoża jest niedoborem złożonym, a „paradoks chlorozy zależnej od żelaza" wynika przynajmniej częściowo z nakładających się na siebie skutków niedoboru żelaza i toksyczności manganu i/lub cynku lub maskowaniu prawidłowego żywienia żelazowego przez toksyczność tychże pierwiastków, ale udział tychże pierwiastków jest specyficzny dla gatunku.

- **Wzrost roślin stenotopowych na niedopasowanym substracie warunkuje silny stres.** Stres powodowany niedoborem żelaza wiąże się z głębokim zachwianiem homeostazy i ma skutki strukturalne (spowolniony wzrost), jak i funkcjonalne (zaburzone żywienie mineralne, metabolizm specjalistyczny i reakcje obronne). Dostępność wapnia i magnezu w glebie zasadowej nie odgrywa znaczącej roli w procesie rozwoju niedoborów żelaza. Rozwój badanych chloroz ma istotny wpływ na pogorszenie funkcjonowania aparatu fotosyntetycznego, choć obserwowane reakcje wskazują na istnienie adaptacji umożliwiających usunięcie nadmiaru energii wzbudzenia na drodze cieplnej. Aktywność enzymatycznego systemu antyoksydacyjnego oraz funkcjonowanie metabolizmu specjalistycznego zależy od typu gleby na której wzrastają rośliny oraz dostępności żelaza. Obserwowane wzorce reakcji wskazują na ostry przebieg stresu u roślin doświadczających głodu żelazowego, co w skrajnych przypadkach powoduje silne zachwianie mechanizmów kontrolujących równowagę oksydoredukcyjną (u kalcyfilów) lub załamanie biosyntezy metabolitów specjalistycznych (u kalcyfobów).

- **Odczyn oraz dostępność żelaza, manganu i glinu pełnią rolę filtrów środowiskowych na wczesnych etapach ontogenezy.** Badane czynniki limitują zdolność nasion roślin występujących w obrębie badanych muraw do wykiełkowania, jednakże żelazo i mangan pełnią rolę drugoplanową w stosunku do odczynu i glinu. Strategie kiełkowania badanych roślin są specyficzne gatunkowo, a progi tolerancji względem badanych czynników są często wyższe niż wynikałoby to z charakterystyki siedlisk, w których dane gatunki występują. Masa diaspor, szacowana preferencja substratowa oraz liczby wskaźnikowe Ellenberga odnoszące się do odczynu gleby nie przejawiają szerszej przydatności jako czynniki predykcyjne w stosunku do strategii kiełkowania (w kontekście badanych czynników edaficznych), co wskazuje istnienie bardziej wyrafinowanych (strukturalno-funkcjonalnych) predyktorów reakcji roślin. Zakres tolerancji względem badanych czynników w fazie kiełkowania jest często szerszy niż zakres tolerancji w późniejszych etapach ontogenezy.

12. Literatura

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13. Oświadczenia

Oświadczenia współautorów
o wkładzie w powstanie publikacji

Oświadczenie współautora o udziale w publikacji

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OŚWIADCZENIE

1) Oświadczam, że w pracy:

Wala, M., Kołodziejek, J., Mazur, J., Patykowski, J., 2020. Differences in iron acquisition strategies of calcicole plant species from xerothermic grasslands. Geoderma 377: 114572. DOI: 10.1016/j.geoderma.2020.114572

*mój udział polegał na: koordynowaniu całości prac, opracowaniu koncepcji pracy i układu doświadczalnego, pozyskaniu materiału (gleb), przeprowadzeniu doświadczenia polowego, zebraniu pomiarów zawartości chlorofilu i danych o fluorescencji chlorofilu a (OJIP), oznaczeniu stopnia chlorozy, wykonaniu pomiarów suchej i świeżej masy roślin, współudziale w wykonaniu pomiarów morfometrycznych liści, przygotowaniu prób do analiz biochemicznych, oznaczeniu aktywności dysmutazy ponadtlenkowej, katalazy i peroksydazy oraz zawartości białka, przygotowaniu prób do analizy zawartości pierwiastków, wykonaniu obliczeń parametrów pochodnych, przeprowadzaniu analizy statystycznej, opracowaniu graficznym wyników (wizualizacja danych), interpretacji wyników, przygotowaniu tekstu pierwszej wersji manuskryptu, przygotowaniu tekstu manuskryptu po rewizji, przygotowaniu merytorycznym i redakcyjnym treści odpowiedzi na recenzje oraz na pełnieniu roli autora korespondencyjnego, co stanowi **75%** ww. pracy.*

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2) *Oświadczam, że w pracy:*

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OŚWIADCZENIE

1) Oświadczam, że w pracy:

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*mój udział polegał na: ocenie postępów prac, opracowaniu koncepcji pracy i weryfikacji merytorycznej układu doświadczalnego, pozyskaniu materiału (nasion), zapewnieniu dostępu do aparatury badawczej, współudziale w oznaczaniu parametrów morfometrycznych liści, weryfikacji poprawności merytorycznej wnioskowania, redakcji tekstu (uwagi krytyczne do pierwszej wersji manuskryptu) oraz weryfikacji zrewidowanej wersji tekstu (uwagi krytyczne do manuskryptu po recenzji), co stanowi **10%** ww. pracy.*

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*mój udział polegał na: ocenie postępów prac, weryfikacji koncepcji pracy i układu doświadczalnego, zapewnieniu dostępu do aparatury badawczej, weryfikacji poprawności merytorycznej wnioskowania, redakcji tekstu (uwagi krytyczne do pierwszej wersji manuskryptu) oraz weryfikacji zrewidowanej wersji tekstu (uwagi krytyczne do manuskryptu po recenzji), co stanowi **10%** ww. pracy.*

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*mój udział polegał na: zapewnieniu dostępu do aparatury badawczej, przygotowaniu opisu metody oznaczeń zawartości pierwiastków, oznaczeniu zawartości pierwiastków (wapnia, magnezu, żelaza, manganu, cynku oraz miedzi) w materiale roślinnym oraz współudziale w przygotowaniu odpowiedzi dla recenzentów, co stanowi **10%** ww. pracy.*

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Mój udział polegał na: przygotowaniu opisu metody syntezy Mn-HBED, syntezie Mn-HBED, charakterystyce Mn-HBED i Fe-HBED metodami spektrofotometrii, FTIR i LC-MS/MS oraz przygotowaniu wykresów przedstawiających wyniki tychże analiz, co stanowi **10%** ww. pracy.

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Wala, M., Kołodziejek, J., Mazur, J., **Cienkowska, A.**, 2021. Reactions of two xeric-congeneric species of *Centaurea* (Asteraceae) to soils with different pH values and iron availability. PeerJ 9: e12417. DOI: 10.7717/peerj.12417

mój udział polegał na: współudziale w oznaczaniu świeżej oraz suchej masy roślin oraz redakcji tekstu (uwagi redakcyjne do pierwszej wersji manuskryptu), co stanowi ***5%*** *ww. pracy.*

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mój udział polegał na: zapewnieniu dostępu do aparatury badawczej, współudziale w przygotowaniu prób do analizy biochemicznej, oznaczaniu aktywności katalazy oraz redakcji tekstu (uwagi krytyczne do pierwszej wersji manuskryptu), co stanowi ***5%*** *ww. pracy.*

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