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The effects of lead and manganese in soil on males and females of the dioecious herb *Spinacia oleracea*

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ABSTRACT

Males and females of dioecious plant species commonly differ in their morphology, physiology or life-history traits, and also in their response to environmental stress, which may lead to sex biases and eventually to population declines. Metal toxicity is an extreme case of environmental stress, where differences in tolerance between males and females have been reported before, but mainly in woody species (trees from the genus *Populus*). Here, we aimed to increase our understanding of the response of the sexes of dioecious species to metal stress by investigating the individual and combined effects of added Pb and Mn to the soil on the growth and physiological performance of male and female plants of the wind-pollinated herb spinach (*Spinacia oleracea* L). We carried out a glasshouse experiment, where male and female plants of *S. oleracea* were grown in soils without and with added Pb, Mn and combined Pb + Mn (added as nitrates), and with different nutrient levels ('low' or 'high' nutrients). The addition of Pb and Mn as nitrates did not have any detrimental effects on the growth, chlorophyll content, %N, %C and C/N in spinach plants. Moreover, the addition of Pb $(NO_3)_2$ and Mn $(NO_3)_2$ appears to have increased the nitrates in the soil, as shown by the improved nitrogen content found in plants growing under low nutrients. Male plants accumulated more Pb and Mn in their aboveground tissues than females, but this was not followed by greater detrimental effects on growth. Overall, this study highlights the relevance of the metal species added in causing toxic effects and points to a greater tolerance to metal stress in males of *S. oleracea*.

1. Introduction

Many dioecious plant species, i.e., where male and female functions are housed in different individuals, show sexual dimorphism beyond their primary sexual traits. Differences between males and females can be expressed in morphological, physiological and life-history traits, and have been commonly attributed to the differential cost associated to reproduction in both sexes (Barrett and Hough, 2013; Obeso, 2002). Resource allocation trade-offs are expected when resources are limiting, so that a greater investment in reproduction by one sex may come at the expense of allocation to other competing functions, such as growth and defence (Obeso, 2002). Environmental stress can accentuate such trade-offs, and hence, we may expect differences between males and females in their response to environmental stress (Juvany and Munné-Bosch, 2015; Retuerto et al., 2018). Differences in performance between the sexes can lead to strong sex-biases, and eventually could lead to population declines or even extinction (Petry et al., 2016). It is generally assumed that females will perform worse than males under stress, due to their greater reproductive costs – producing not only flowers but also fruits (Barrett and Hough, 2013; Obeso, 2002). However, there are some species, particularly wind-pollinated herbs, where males invest considerably more resources – particularly in terms of nitrogen – in producing pollen (Harris and Pannell, 2008). There is, therefore, not a universal pattern in the response of the sexes to environmental stress, and differences in tolerance seem to vary greatly depending on the species, its life history (e.g., trees versus herbs), and type of stress, which demands a greater range of studies (Juvany and Munné-Bosch, 2015).

Soil pollution by metals, mainly due to anthropogenic activities (e.g., industrial activities, agricultural use of fertilizers and pesticides), is an extreme type of environmental stress that is currently a worldwide problem. Metals can be considered environmental pollutants due to their strong toxic effects, threatening the environment and human health (Zhao et al., 2022). Metals such as manganese (Mn), are

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'essential', functioning as a cofactor in key biological processes, such as photosynthesis, lipid biosynthesis and the regulation of reactive oxygen species (ROS) through Mn-dependent antioxidant enzymes, helping to protect plants from oxidative damage (Alejandro et al., 2020; Socha and Guerinot, 2014). However, when present in excess, Mn disrupts cellular homeostasis by triggering ROS overproduction, which leads to oxidative stress and damages key physiological process such as chlorophyll biosynthesis and photosynthesis, while also interfering in the uptake and translocation of other essential minerals (phosphorous (P), magnesium (Mg) and iron (Fe)) (Lei et al., 2007; Millaleo et al., 2010, 2018). Mn toxicity typically manifests as chlorosis and tissue necrosis, which ultimately leads to a decrease in plant growth and biomass (Socha and Guerinot, 2014). Lead (Pb) is an example of a 'non-essential' metal—i.e., toxic even in trace amounts—that has been described as one of the most toxic heavy metals, second only to arsenic (As), a highly toxic metalloid (Jaishankar et al., 2014; Zulfiqar et al., 2019). Plants exposed to Pb, even in small amounts, are adversely affected by disruption of key physiological processes, such as reduction in photosynthesis, impaired mineral nutrition and water imbalance, and the inhibition of chlorophyll synthesis and enzyme activities, causing oxidative stress (Aslam et al., 2020; Sharma and Dubey, 2005). Typical symptoms of Pb toxicity are stunted growth, inhibition of root growth, and chlorosis, and ultimately, Pb exposure can lead to plant death (Sharma and Dubey, 2005).

The tolerance of plants to metal stress may vary depending on several factors, including metal species and concentration, the length of exposure, but also plant species, the growth stage of the plant, or even due to individual variation within species (Viehweger, 2014; Wang et al., 2007). In fact, male and female plants of dioecious species have been found to differ in their tolerance to metal stress, with males usually having greater tolerance than females (F. Chen et al., 2013; Chen et al., 2011; L. Chen et al., 2013; Han et al., 2013; Jiang et al., 2013), but see Sánchez Vilas et al. (2016). However, despite the widespread pollution of soils with metals, there is still a scarcity of studies investigating the effects on the cost of reproduction and the response of the sexes to metal stress, with studies mainly limited to woody species, particularly trees from the genus *Populus* (Yu et al., 2023). Therefore, here, we aimed to increase our understanding of the response of the sexes of dioecious species to metal stress, by investigating the individual and combined effects of added Pb and Mn to the soil on the growth and performance of male and female plants of the wind-pollinated herb spinach (*Spinacia oleracea* L). We hypothesise that elevated concentrations of Pb and Mn in the soil, individually or in combination, will negatively affect the growth and physiological performance of spinach plants, and that male and female plants will differ in their tolerance to metal stress.

2. Materials and methods

2.1. Study species

Spinach, *Spinacia oleracea* L. (Chenopodiaceae), is an annual (or biannual), wind-pollinated herb that is widely cultivated worldwide as a leafy vegetable. Spinach is widely known as dioecious, i.e., with male and female reproductive functions in separate plants, and has long been recognised as a valuable model for studying dioecy, sex determination, and sexual dimorphism in plants (Pérez-Llorca and Sánchez Vilas, 2019; Yamamoto et al., 2014). It was among the first species in which dimorphism between sexual morphs was investigated (Onyekwelu and Harper, 1979) and in recent years has been used extensively for exploring mechanisms of sex determination and sex chromosome evolution (Ma et al., 2022; Onodera et al., 2011; She et al., 2025, 2024; Yamamoto et al., 2014; You et al., 2025; Zhao et al., 2025).

2.2. Experimental design

Seeds of *Spinacia oleracea* L. var Medania were purchased from Chiltern Seeds (Wallingford, UK) and sown in germination trays containing a 2:1 substrate mixture of sand (Horticultural Sand, Westland Horticulture Ltd, Northern Ireland) to compost (John Innes No 2, Westland Horticulture Ltd., Northern Ireland). Nineteen days after germination, 186 seedlings were transplanted into 0.3 L pots and randomly allocated to the different experimental treatments, with plants growing under different heavy metals ('no metal', 'Pb', 'Mn' and 'Mn + Pb') and under different nutrient levels ('low' (LN) and 'high' (HN) nutrients). Pb and Mn were added to a sandy soil poor in nutrients (3:1 sand to compost, pH = 7.3), mixed and left for a week prior to the start of the experiment. 0.1 M $Pb(NO_3)_2$ solution (870 mL) was added to 32 kg of substrate to achieve a soil concentration of 900 mg of $Pb(NO_3)_2$ kg^{-1}. 0.1 M $Mn(NO_3)_2$ solution (1.79 L) was added to the 32 kg of substrate to achieve a soil concentration of 1000 mg of $Mn(NO_3)_2$ kg^{-1}. For the combined Mn + Pb treatment, 870 mL of 0.1 M $Pb(NO_3)_2$ solution and 1.79 L of 0.1 M $Mn(NO_3)_2$ solution were added to 32 kg of soil. High levels of nutrients (HN) were applied by adding to each pot 1.5 g of slow-release fertiliser (MiracleGro All Purpose, NPK 24–8–16, OMS Investments Ltd, London, UK), whilst pots with low nutrients were not fertilised (LN). Plants were watered ad libitum for the duration of the experiment. Containers were placed under each pot to prevent leaching during watering, and plants were placed at random on the benches of a glasshouse with an average temperature of 23.4 ± 0.5 °C, average relative humidity of 54.6 ± 1.1 % and with a photoperiod of 12 h day/12 h night in the Plant Growth Facilities of Cardiff University. Artificial lighting was supplied by high-intensity lamps providing about 250–300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD (Photosynthetic Photon Flux Density) at the level of the plants.

2.3. Plant growth and chlorophyll content

Plants were grown for 48 days (from 16th December 2021 to 2nd February 2022) under experimental treatments, harvested, dried at 60 °C for seven days until reaching a constant dry weight and their aboveground (leaves/stems/flowers) dry mass was measured (± 0.0001 g; Mettler Toledo). Prior to harvest, the chlorophyll content of three fully newly developed leaves per plant was measured with a hand-held chlorophyll meter (SPAD-502, Konica Minolta Inc., Japan), which calculates an index based on absorbance at 650 and 940 nm. SPAD values are well correlated with the chlorophyll content of leaves (Gamon and Surfus, 1999; Richardson et al., 2002). At harvest, the sex of the flowering plants was recorded by assessing the presence of male or female flowers. There were 3 casualties of unknown sex, belonging to the 'Pb' low and high nutrients, and to 'Mn + Pb' low nutrients. Given that sex could only be assessed at harvest we ended up with unequal sample size for males and females allocated to each treatment (for details regarding sample size see Fig. 1).

2.4. Nitrogen and carbon contents, and C/N ratio in the aboveground plant biomass

Nitrogen content per mass (N_{mass}, %), carbon content per mass (C_{mass}, %) and the C/N ratio were determined for a randomly chosen subset of 5 plants for each sex growing under the different experimental treatments [(5 males + 5 females) × 4 Metal Treatments × 2 Nutrient Treatments = 80 replicates]. A subsample of each dried plant (≈100 mg) was ground to a fine powder (<1 μm) and sent for analysis with a Thermo Flash EA 1112 elemental analyser in the School of Earth and Environmental Sciences (Cardiff University).

2.5. Pb and Mn analysis

The dry aboveground parts were sent to ALS Life Sciences Ltd. (Deeside, UK) for Pb and Mn analysis. Hydrochloric acid (7.5 mL) and nitric acid (2.5 mL) were mixed and used to digest 1 g plant material (n = 3 repeats per sample) for 90 mins at 110 °C. The solution was subsequently diluted to 50 mL using de-ionised water and centrifuged at

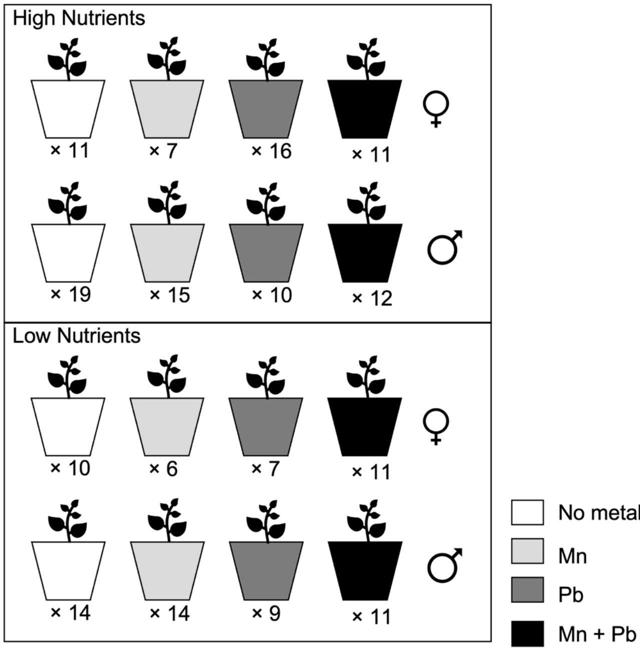

Fig. 1. Schematic representation of the experimental design consisting of pots with males and females of *Spinacia oleracea* growing in soil with high nutrients and low nutrients and under different metal treatments (No metal, Mn, Pb and Mn + Pb). The numbers below the pots indicate the number of replicates per nutrient and metal combination. Because sex could only be determined at the time of harvest, we were unable to assign individuals to treatments based on sex in advance. As a result, the number of males and females per treatment was unequal.

1500 rpm for 15 mins. The supernatant was transferred to a vial and analysed by ICP-OES (ICAP 65,000 Duo, Thermo Scientific). To fulfil the requirements of sample size for analysis, two samples per treatment were grouped for the analysis of Pb and Mn. As a result, there were 4 replicates per sex and experimental treatment, except for females growing under Mn (LN and HN) and under Pb LN where only 3 replicates were available (total replicates = 61). For Pb analysis, treatments without added Pb gave values below the detection limit of the instrument, and hence the results refer only to comparison of the metal treatments Pb and Mn + Pb.

2.6. Data analysis

All data were analysed using R version 4.0.0 (R Core Team, 2022). The effects of the experimental treatments on aboveground dry mass, chlorophyll content, C, N, and C/N and Pb and Mn were determined by means of analysis of variance, using the 'lm' function in R with *sex* (male, female), *metal* (no metal, Pb, Mn and Mn + Pb) and *nutrients* (HN, LN) and their interactions as fixed factors. Models were assessed visually for assumption of normality and homoscedasticity; N was squared, and the C/N ratio was square root transformed to meet the assumptions of the analysis of variance. *P* values were obtained by calling the Anova function in the 'car' package, using sum of squares type 'III'. Post-hoc testing was then carried out using Tukey HSD method through the 'emmeans' package.

3. Results

3.1. Plant growth and chlorophyll content

The addition of nutrients increased the aboveground dry mass of plants, and this increase was greater for females than males (Table 1, Fig. 2), with differences appearing between the sexes when growing under high nutrients (Fig. 2a; females were significantly larger than males). There was an interaction between the metal and the nutrient treatments (Table 1), the addition of nutrients increased the aboveground dry mass of the plants but only in the absence of metals. If metals were added, no differences were found between low and high nutrients in the aboveground dry mass (Fig. 2b).

Females had greater values of chlorophyll content (SPAD values) than males, regardless of metal and nutrient treatments (Table 1, mean ± SE: 32.9 ± 0.6 for females and 28.0 ± 0.6 for males). The addition of nutrients also increased the chlorophyll content (SPAD values) of the plants; however, this depended on the metal treatment (Table 1; significant interaction metal × nutrients). Plants growing under no metal, or under Mn and Pb addition increased their chlorophyll content in response to the addition of nutrients, but no response was found when plants were exposed to both Mn + Pb (Fig 2c).

3.2. Nitrogen and carbon contents, and C/N ratio in the aboveground plant biomass

Females had greater % N than males in their tissues (Table 2, mean ± SE: 5.11 ± 0.19 for females and 4.80 ± 0.15 for males). Nutrient availability significantly influenced the percentage of nitrogen (% N) in the aboveground dry mass of the plants, but this effect varied depending on the metal treatment (Table 2; significant interaction metal × nutrients). Specifically, plants grown in soils without added metals showed a decrease in % N in their aboveground tissues when exposed to low nutrient conditions. However, when plants grew in soil with Mn, Pb or Mn + Pb their % N in aboveground tissues remained similar in both nutrient treatments (Fig. 3a). Overall, the % C in aboveground tissues was greater in females than in males (Table 2, sex, mean ± SE: 34.97± 0.30 for females and 32.03 ± 0.46 for males), but % C was not affected significantly by the different metal and nutrient treatments (Table 2). The C/N ratio was significantly affected by the interaction metal x nutrient, with those plants growing under 'no metal' increasing their C/N ratio when growing under low nutrient availability. However, this increase was not found when metals were added to the soil (Fig. 3b).The C/N ratio differed between the sexes in response to the addition of metals (Table 2, significant interaction sex x metal):females decreased

Table 1
Analysis of variance for aboveground dry mass (g) and chlorophyll content (SPAD units) of male and female plants of *Spinacia oleracea* growing under different metal (no metal, Mn, Pb and Mn+Pb) and different nutrient availability (Low and High Nutrients). Significant *P* values (*P* < 0.05) are highlighted in bold.

Parameter	Aboveground dry mass (g)				Chlorophyll content (SPAD units)			
	df	SS	F	*P*	df	SS	F	P
Sex	1	3.939	31.006	**< 0.0001**	1	1,034	34.062	**< 0.0001**
Metal	3	0.255	0.670	0.571	3	99	1.084	0.357
Nutrients	1	5.479	43.128	**< 0.0001**	1	1,181	38.92	**< 0.0001**
Sex × Metal	3	0.902	2.368	0.073	3	137	1.503	0.216
Sex × Nutrients	1	0.588	4.627	**0.033**	1	17	0.544	0.462
Metal × Nutrients	3	1.471	3.860	**0.011**	3	288	3.159	**0.026**
Sex × Metal × Nutrients	3	0.284	0.744	0.527	3	100	1.093	0.354
Error	167	21.214			167	5,068		

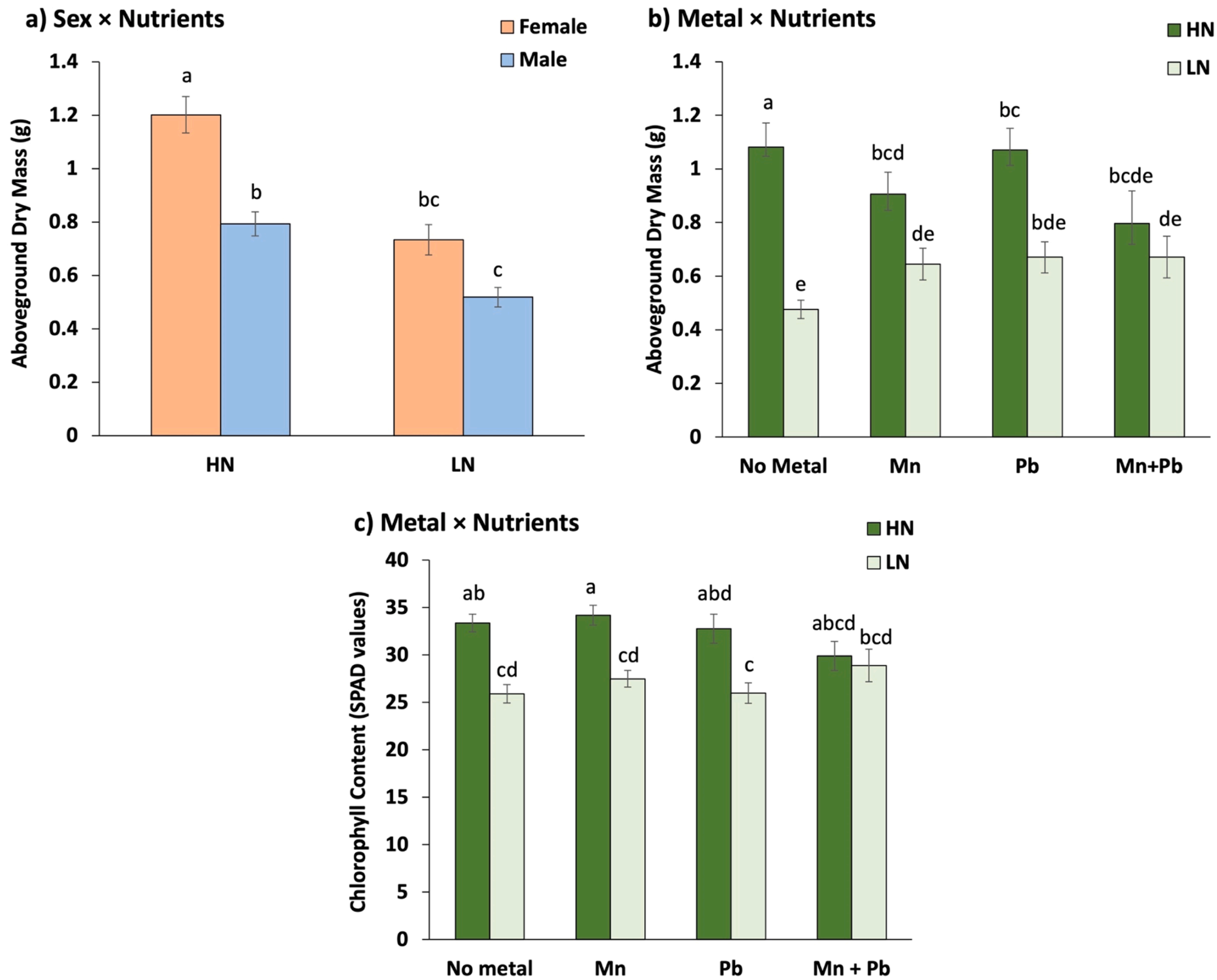

Fig. 2. Aboveground dry mass (a) of males and females of *Spinacia oleracea* in response to growing in soil with different nutrient availability (HN: high nutrients, LN: low nutrients) and aboveground dry mass (b) and chlorophyll content (c) of *S. oleracea* plants in response to growing in soil with different metal treatments (No metal, Mn, Pb and Mn + Pb) and with (HN) or without (LN) added nutrients. Bars and error bars represent means and S.E., respectively, based on raw data. For *N* see Fig. 1. Different letters above bars indicate significant differences between groups ($P < 0.05$, Tukey's HSD test).

Table 2
Analysis of variance for the percentage of N and C per mass of aboveground dry mass and for the C/N ratio of male and female plants of *Spinacia oleracea* growing under different metal (no metal, Mn, Pb and Mn+Pb) and different nutrient availability (Low and High Nutrients). Significant *P* values ($P < 0.05$) are highlighted in bold.

Parameter		% N			%C			C/N		
	df	SS	F	*P*	SS	F	P	SS	F	*P*
Sex	1	261	4.132	**0.046**	173	28.043	**<0.0001**	0.13	1.164	0.285
Metal	3	1,258	6.643	**< 0.001**	1	0.047	0.986	1.51	4.585	**0.006**
Nutrients	1	1,443	22.860	**< 0.0001**	21	3.333	0.073	2.68	24.403	**< 0.0001**
Sex × Metal	3	426	2.248	0.091	13	0.681	0.567	0.98	2.969	**0.038**
Sex × Nutrients	1	58	0.912	0.343	5	0.874	0.353	0.13	1.228	0.272
Metal × Nutrients	3	532	2.811	**0.046**	12	0.645	0.589	1.32	4.002	**0.011**
Sex × Metal × Nutrients	3	79	0.419	0.740	16	0.882	0.455	0.15	0.449	0.719
Error	64	4,039			396			7.02		

their C/N ratio in response to growing with added Pb, but the C/N ratio of males was not affected in response to metals(Fig. 3c).

3.3. Pb and Mn concentrations

Males had greater concentration of Mn in their aboveground tissues than females (Table 3, mean ± SE: 71.99 ± 8.67 for females and 107.70 ± 12.46 for males), regardless of the nutrient and metal treatment. Spinach plants growing in soil with added Mn (both the 'Mn' and the 'Mn + Pb' treatments) increased the concentration of Mn in their aboveground tissues. However, this increase was only significant depending on the nutrient availability (Table 3, significant interaction metal treatment × nutrient): under 'Mn' growing in high nutrients, and under 'Mn + Pb' growing under low nutrients (Fig. 4).

The concentration of Pb in the aboveground tissues of spinach plants ranged from 26.25 ± 16.7 to 90.8 ± 8 mg/kg and was significantly affected by the third order interaction 'sex × metal × nutrients' (Table 3, Fig. 5). Particularly, no differences in the concentration of Pb were found between males and females when plants were growing under high nutrients; however, when growing under low nutrients (LN), males

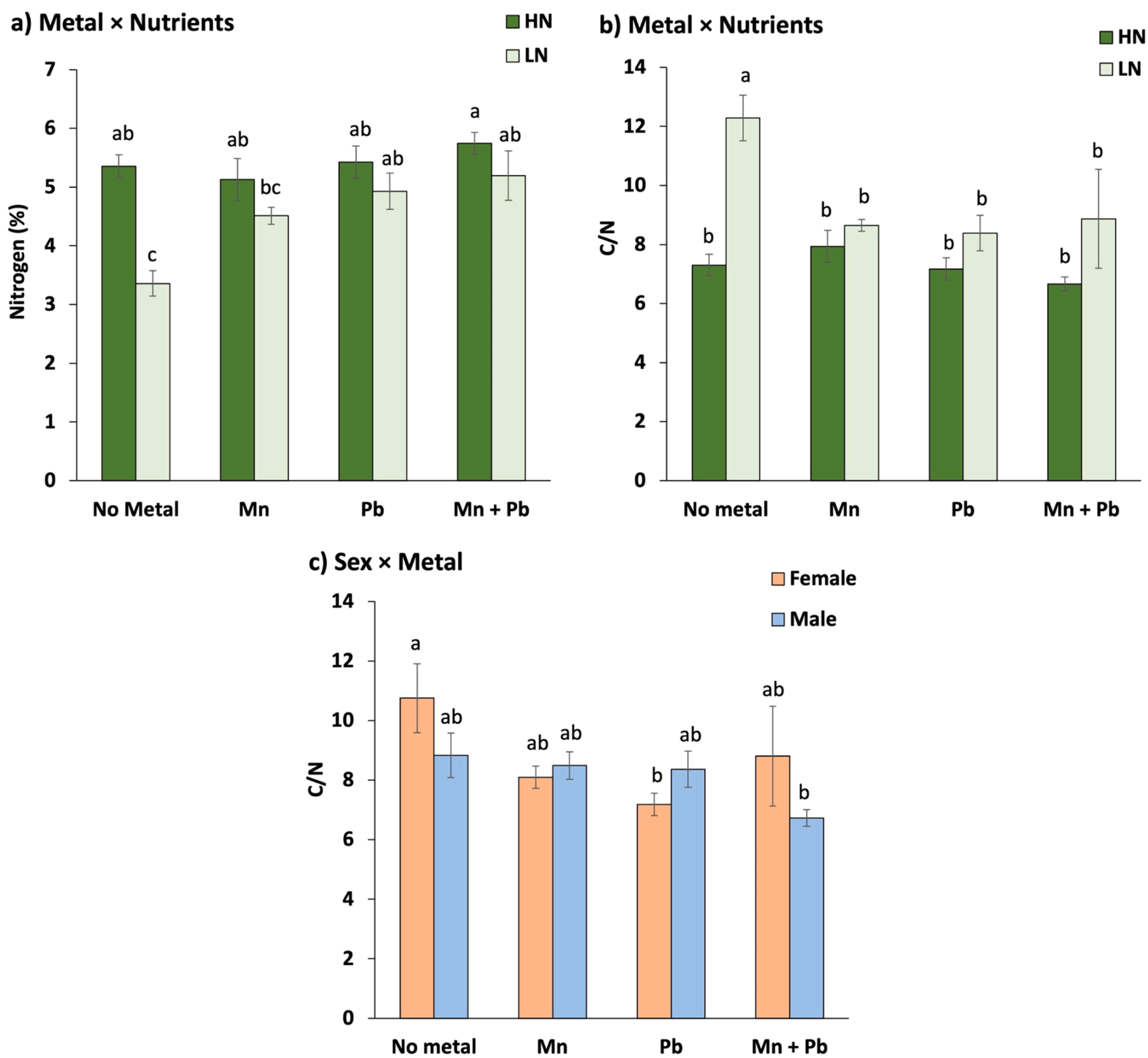

Fig. 3. %N (a) and C/N ratio (b) of *Spinacia oleracea* plants in response to growing in soil with different metal treatments (No metal, Mn, Pb and Mn + Pb) and with (HN) or without (LN) added nutrients and C/N ratio (c) of males and females of *S. oleracea* in response to growing in soil with different metal treatments. Bars and error bars represent means and S.E., respectively, based on raw data ($N = 10$). Different letters above bars indicate significant differences between groups ($P < 0.05$, Tukey's HSD test).

Table 3
Analysis of variance for the concentration of Mn (mg/kg) and Pb (mg/kg) in the aboveground tissues of male and female plants of *Spinacia oleracea* growing under different metal (no metal, Mn, Pb and Mn+Pb) and different nutrient availability (Low and High Nutrients). Significant *P* values ($P < 0.05$) are highlighted in bold. Note that for the concentration of Pb (mg/kg), data refer only to two metal treatments, Pb and Pb +Mn (hence the different df), as the treatments without added Pb were below the detection limit of the instrument.

Parameter	Mn (mg/kg)				Pb (mg/kg)			
	df	SS	F	*P*	df	SS	F	P
Sex	1	19,251	10.621	**0.002**	1	2,273	3.687	0.067
Metal	3	90,505	16.644	**<0.0001**	1	4,660	7.559	**0.011**
Nutrients	1	36	0.020	0.889	1	415	0.674	0.420
Sex × Metal	3	8,652	1.591	0.205	1	2,623	4.254	0.051
Sex × Nutrients	1	3,736	2.061	0.158	1	278	0.451	0.509
Metal × Nutrients	3	17,175	3.158	**0.034**	1	15	0.024	0.878
Sex × Metal × Nutrients	3	9,245	1.700	0.180	1	3,093	5.016	**0.035**
Error	45	81,563			23	14,179		

growing in soil with added Pb accumulated significantly more metal than females growing in the same soil, and those males growing in soil with both Mn + Pb (Fig. 5).

4. Discussion

Overall, in our study, the growth of spinach plants was unaffected by

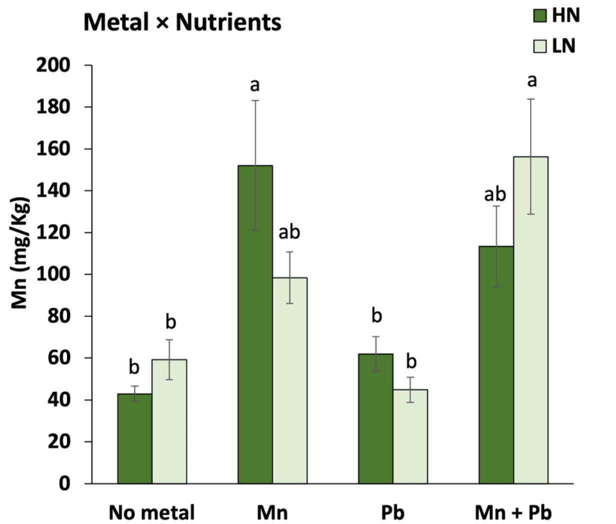

Fig. 4. Concentration of Mn in aboveground dry mass (mg.kg^{-1}) of *Spinacia oleracea* plants in response to growing in soil with different metal treatments (No metal, Mn, Pb and Mn + Pb) and with (HN) or without (LN) added nutrients. Bars and error bars represent means and S.E., respectively, based on raw data (No metal: $N_{HN} = 8$, $N_{LN} = 8$; Mn: $N_{HN} = 7$, $N_{LN} = 7$; Pb: $N_{HN} = 8$, $N_{LN} = 7$; Mn + Pb: $N_{HN} = 8$, $N_{LN} = 8$). Different letters above bars indicate significant differences between groups ($P < 0.05$, Tukey's HSD test).

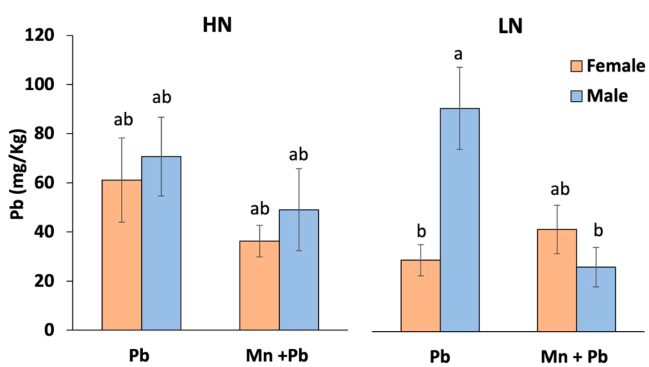

Fig. 5. Concentration of Pb in aboveground dry mass (mg.kg^{-1}) of males and females of *Spinacia oleracea* in response to growing in soil with different metal treatments (Pb and Mn + Pb) and with (HN) or without (LN) added nutrients. Bars and error bars represent means and S.E., respectively, based on raw data ($N = 4$, except for females growing under Pb in LN where $N = 3$). Different letters above bars indicate significant differences between groups ($P < 0.05$, Tukey's HSD test).

the addition of the metals. In general, plants differ considerably in their susceptibility to excess metals, with critical toxic concentrations that can range greatly depending on factors such as the plant species, or other factors such as temperature (Reichman, 2002). For Mn, critical toxicity concentrations have been reported ranging from 200 mg.kg^{-1} to 5300 mg.kg^{-1} dry mass (Dučić and Polle, 2005; Edwards and Asher, 1982). In our study, we found that tissue concentrations for Mn were always below 200 mg.kg^{-1}, which may explain the lack of detrimental effects on plant growth. For Pb, however, the results were unexpected; Pb is a non-essential metal and in our study, Pb accumulated in the spinach tissues in ranges (11–105 mg.kg^{-1}) that have been identified as toxic for plants (Fahr et al., 2013). Hence, spinach appears to have tolerance to Pb concentrations below and up to 105 mg.kg^{-1} in its aboveground tissues. Hyperaccumulator plants can tolerate concentrations much higher than those values, reaching concentrations above 2000 mg.kg^{-1} in their leaves and shoots without significant damage, and can be used for phytoremediation of Pb-contaminated soil (Gupta et al., 2013). In the case of spinach, this tolerance may pose health risks, particularly if apparently healthy plants are consumed by humans (Rusin et al., 2021).

As expected, the addition of nutrients improved the growth and chlorophyll content of plants. Interestingly, the addition of metals as nitrates appears to have improved the nitrogen status of plants; under no metals, there was a clear reduction in the nitrogen content when plants grew under low nutrients, but their nitrogen content improved when metals were added to the soil. Adding lead and manganese as nitrate species could increase the concentration of nitrate in the soil, a major source of nitrogen for plants, and this could alleviate the potential toxic effects associated to the added metals. However, this should be further investigated, and little research has considered how different metal species influence plants' tolerance. There are only a few studies that have found that increasing the concentration of $Pb(NO_3)_2$ enhanced plant growth and nutritional composition (Corley and Mutiti, 2017; Pracheta et al., 2009).

The response of the plant sexes to the addition of metals did not differ in terms of plant growth or chlorophyll content; however, overall, females grew more than males. Differences in size between females and males have been reported before, and a similar pattern has been found for *S. oleracea* and for other wind-pollinated dioecious species, such as *Mercurialis annua*, and have been attributed to the different resource demands associated with reproduction (Harris and Pannell, 2008; Pérez-Llorca and Sánchez Vilas, 2019; Sánchez Vilas et al., 2016). Females also had higher chlorophyll content and nitrogen in their aboveground tissues than males, which again can be attributed to differences in resource demand. In wind pollinated plants, such as *S. oleracea*, males are found to require more nitrogen for reproduction – due to producing high amounts of N-rich pollen – whilst females require more carbon for seed development (Barrett and Hough, 2013; Harris and Pannell, 2008). Despite no differences in growth or chlorophyll content in response to metals, the C/N ratio of females was significantly reduced when growing under Pb addition, whilst it remained unaffected in males, which also could suggest different strategies of resource allocation (Chen et al., 2017; Rabska et al., 2020). Interestingly, we did find differences in the accumulation of metals between males and females, with males accumulating higher concentrations of Mn and Pb than females in their aboveground tissues. Differential accumulation of metals in males and females of dioecious species has been found before, and has been attributed to differences in uptake, translocation, distribution and detoxification of metals between males and females (Lin et al., 2022). Differences in reproductive demands between the sexes have been invoked to explain differences in accumulation of metals; resource demands associated with male and female functions can lead to sex-specific trade-offs between investment in reproduction, growth and responses to stress, including mechanisms involved in accumulation and tolerance to heavy metals (Juvany and Munné-Bosch, 2015; Retuerto et al., 2018; Xu et al., 2016). Since no detrimental effect was seen in males because of a higher accumulation, this may indicate that spinach males have a greater tolerance to these heavy metals than females. Indeed, there is some evidence confirming our results, of males accumulating and having greater tolerance to metals; however, most of these findings come from woody trees, particularly from the genus '*Populus*' (L. Chen et al., 2013; Han et al., 2013; Lei et al., 2007; Xu et al., 2016; Zhang et al., 2010).

5. Conclusion

The addition of Pb and Mn as nitrates did not have any detrimental effects on the growth, chlorophyll content, % N and % C in spinach plants. Moreover, the addition of $Pb(NO_3)_2$ and $Mn(NO_3)_2$ appears to have increased the nitrate concentration in the soil, as shown by the improved nitrogen content found in plants growing under low nutrients. Female plants grew more and had higher chlorophyll and nitrogen content than male plants, regardless of the metal treatment. However, the sexes differed in the accumulation of metals, with males accumulating more Pb and Mn in their aboveground tissues, pointing to a greater tolerance to metal stress than females. Overall, this study highlights the relevance of the metal species added in causing toxic effects, and points to a greater tolerance to metal stress in males of *S. oleracea*.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The data used to support the findings of this study are available from the authors upon request.

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Authors' contributions

All authors contributed to the design of the experiment, MF and JSV collected and analysed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

CRediT authorship contribution statement

M. Fisher: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **K. BéruBé:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Conceptualization. **T. Jones:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Conceptualization. **B. Rees:** Writing – review & editing, Resources, Methodology. **J. Sánchez Vilas:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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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Data availability

Data will be made available on request.

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