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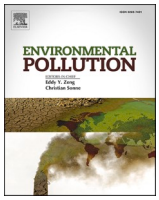

Arbuscular mycorrhizas amplify the risk of heavy metal transfer to human food chain from fly ash ameliorated agricultural soils☆

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ABSTRACT

Soil contaminants threaten global food security by posing threats to food safety through food chain pollution. Fly ash is a potential agent of soil contamination that contains heavy metals and hazardous pollutants. However, being rich in macro- and micronutrients that have direct beneficial effects on plant growth, fly ash has been recommended as a low-cost soil ameliorant in agriculture in countries of the Global South. Arbuscular mycorrhizal fungi (AMF), ubiquitous in agricultural soils, enhance efficiency of plant nutrient uptake from soils but can equally increase uptake of toxic pollutants from fly ash ameliorated soils to edible crop tissues. We investigated AMF-mediated amplification of nutrient and heavy metal uptake from fly ash amended soils to shoots, roots and grains of barley. We used a microcosm-based experiment to analyse the impacts of fly ash amendments to soil in concentrations of 0 (control), 15, 30 or 50% respectively, on root colonization by AMF *Rhizophagus irregularis* and AMF-mediated transfer of N, P and heavy metals: Ni, Co, Pb and Cr to barley tissues. These concentrations of fly ash are equivalent to 0, 137, 275 and 458 t ha^{-1} respectively, in soil. Root AMF colonization correlated negatively with fly ash concentration and was not detected at 50% fly ash amendment. Shoots, roots and grains of mycorrhizal barley grown with 15, 30 and 50% fly ash amendments had significantly higher concentrations of Ni, Co, Pb and Cr compared to the control and their respective non-mycorrhizal counterparts. Presence of heavy metals in barley plants grown with fly ash amended soil and their increased AMF-mediated translocation to edible grains may significantly enhance the volume of heavy metals entering the human food chain. We recommend careful assessment of manipulation of agricultural soils with fly ash as heavy metal accumulation in agricultural soils and human tissues may cause irreversible damage.

1. Introduction

Soil contaminants threaten global food security by posing threats to food safety due to food chain pollution (Kopittke et al., 2019; Hou et al., 2020). Fly ash is a potential agent of soil contamination that contains heavy metals (e.g. Ni, Co, Pb, Cr, Hg, Cd), hazardous organic pollutants (e.g. carcinogenic polyaromatic hydrocarbons and polychlorinated biphenyls) and radionuclides such as ^{238}U, ^{226}Ra, ^{232}Th, ^{40}K and ^{210}Po (Sahu et al., 2009, 2014; Meer and Nazir, 2018; Jambhulkar et al., 2018; Singh et al., 2023). It is a human-made industrial by-product of coal combustion in thermal power plants and other coal and biomass burning industries. Global annual production of fly ash is 800 million tonnes per year and is expected to increase to 2100 million tonnes by 2032 (Song et al., 2020). Depending on the source and type of coal, fly ash is rich in macro- and micronutrients which have direct beneficial effect on plant growth. When applied to soil, fly ash acts as a conditioner by altering soil texture, nutrient content, cation exchange and water retention (Channabasava et al., 2015; Yao et al., 2015; Yadav and Pandita, 2019). Due to these qualities, and the need to economically dispose of the large quantities generated by thermal power plants and other coal burning industries, fly ash has been recommended as a low-cost soil ameliorant in agriculture in countries of the Global South (Ukwattage et al., 2013; Dash et al., 2015).

Fly ash amended agricultural soils will invariably contain arbuscular

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mycorrhizal fungi (AMF) due to their ubiquitous distribution. These fungal symbionts form mutualistic association with 90% of agricultural crops, particularly cereals and vegetables (Posta and Duc, 2019; Diagne et al., 2020). AMF extraradical mycelia increase the root absorptive surface area of crops (Smith and Read, 2010). This enhances efficiency of elemental ion uptake from the soil solution to crop tissues (Diagne et al., 2020). Crops grown on fly ash amended agricultural soils can absorb heavy metals and other pollutants present in fly ash (Yan et al., 2020; Taupedi and Ultra, 2022). AMF can amplify this absorption. Direct and AMF mediated uptake of pollutants such as heavy metals present in fly ash by crop tissues and their localization in edible parts like grains and leaves can potentially transmit these pollutants to the human food chain (Watts-Williams and Gilbert, 2021). Consequently, fly ash application to agricultural soils must be assessed from a societal and human nutrition perspective.

The concentrations at which fly ash amendments are added to soils cause a manifold increase in amounts of nutrients as well as pollutants like heavy metals available for uptake by plants. For instance, in India fly ash is applied to agricultural fields in concentrations of 12–640 t ha^{-1} (Kumar et al., 2005; Sheoran et al., 2014; Varshney et al., 2022). At 458 t ha^{-1} concentration, fly ash amended soils contain 12 and 1.6 times more N and P respectively, than without fly ash amelioration. Moreover, at this concentration, the amounts of heavy metals such as Ni, Co, Pb and Cr are 25, 49, 27 and 87 times higher respectively, than in soils without fly ash amendment (Supplementary S1).

Despite evidence that AMF enabled nutritional flow to the host plant may not differentiate between nutrient and metal ions (Smith and Read, 2008; Chen et al., 2017; Kabir et al., 2020), agricultural practices employing fly ash amendments to soil have ignored the danger of these fungi amplifying the risk of pollutant transfer from fly ash amended soil to edible parts of the crop. To the best of our knowledge, no study has investigated the implications of AMF mediated increase in heavy metal uptake from fly ash amended soils to human nutrition. Here, using barley (*Hordeum vulgare*) as a crop-model, we investigated how arbuscular mycorrhizas amplify risk of heavy metal transfer to the human food chain by increasing the concentrations of heavy metals Ni, Co, Pb and Cr respectively in grains of barley grown on fly ash amended soils. We selected Ni, Co, Pb and Cr for analyses as representative of the heavy metals present in fly ash. Ni and Co are considered beneficial for plants due to their role as cofactors of enzymes involved in oxidative stress response, metabolism and signalling (Fabiano et al., 2015; Sule et al., 2020; Hu et al., 2021). Ni is also a known carcinogen (Tchounwou et al., 2012). Ni and Co exposure is a public health concern as both their long-term exposure and deficiency can adversely affect human health. Cr and Pb are potential human carcinogens and systemic toxicants causing multiple organ damage even at low levels of exposure. They are also amongst the priority heavy metals of great public health concern due to their high degree of toxicity (Tchounwou et al., 2012).

2. Materials and methods

2.1. Plant and fungal materials

Barley (*H. vulgare*) variety DWRB160 Karan Maltsona and *Rhizophagus irregularis* MUCL 41833 were used as the host plant and mycorrhizal inoculum, respectively.

2.2. Plant growth substrate

Loam soil amended with fly ash in concentrations of 0, 15, 30 or 50% (w/w) respectively was used as the plant growing substrate. These concentrations are equivalent to 0, 137, 275 and 458 t ha^{-1} fly ash in soil. Freshly deposited fly ash (1–4 days old) was collected from fly ash dumps of the National Thermal Power Corporation power plant at Badarpur, New Delhi (28°31′10.31″N, 77°19′25.37″E), India. Chemical characteristics of the soil and fly ash used in the experiment are provided in Supplementary S2. The soil-fly ash mixtures were autoclaved at 121 °C for 2 h. Around 440 g autoclaved soil-fly ash mixtures prepared above (0, 15, 30 or 50% fly ash amendments) were taken in 500 mL microcosms (diameter 10 cm). Each microcosm received 10 g of viable *R. irregularis* inoculum or autoclaved *R. irregularis* inoculum (121 °C; 2 h) that contained ~214 spores. The inoculum was overlain with ~2 cm of autoclaved 0, 15, 30 or 50% fly ash amended soil such that the final weight of each microcosm was 500 g. Microcosms that received the viable inoculum were labelled as mycorrhizal and those that received autoclaved inoculum were labelled as non-mycorrhizal. Mycorrhizal and non-mycorrhizal microcosms were given the following four fly ash treatments: (i) 0% fly ash; (ii) 15% fly ash; (iii) 30% fly ash and (iv) 50% fly ash. Microcosms with 0% fly ash served as the control. With 10 replicates for each mycorrhizal or non-mycorrhizal fly ash treatment, there were a total of 80 microcosms.

2.3. Microcosm experiment

H. vulgare seeds were surface sterilized with 0.1% $HgCl_2$ for 5 min and washed with sterile double deionized water. Surface sterilized seeds were planted in autoclaved soil (121 °C for 2 h). Following germination, five-day old seedlings were transferred to the microcosms (five seedlings per microcosm) prepared above.

To correct for possible differences in bacterial and non mycorrhizal fungal communities, each non-mycorrhizal microcosm received 10 mL inoculum wash (Koide and Li, 1989) prepared as described by Deepika and Kothamasi (2015). The plants were grown at ambient temperature (20–40 °C) under a day-night cycle of 16–8 h and were fertilized every 15 days with 10 mL Hoagland solution (Hoagland and Arnon, 1950) containing half of the normal P concentration. Microcosms were randomized once every seven days.

2.4. Harvest

Plants were harvested after 16 weeks of growth. Soil-fly ash substrate adhering to the roots was removed by washing thoroughly under tap water. The plants were separated into shoots, roots and grains and weighed to measure fresh weight. A sub sample of the roots was set aside for determination of AMF colonization. Shoots, roots and grains were dried at 70 °C for five days and weighed again to determine dry weight. Dry weights of root subsamples were corrected to account for the removed subsamples.

2.5. AMF colonization of roots

Roots were cleared in 10% KOH at 90 °C for ~30 min and stained with 0.05% trypan blue. The percentage of root length colonized with AMF arbuscles, vesicles and hyphae were determined using the modified line intersection method (McGonigle et al., 1990). Thirty 1 cm root segments were analysed from each pot and 200 intersections were counted in each segment.

2.6. Mycorrhizal species sensitivity of barley

Mycorrhizal species sensitivity is the variation in growth response of a plant species when associated with different AMF species and is calculated as the coefficient of variation on the dry mass in response to each AMF species (van der Heijden, 2002; Klironomos, 2003). The literature in mycorrhizal ecology often equates sensitivity to AMF with species specificity but plant sensitivity to AMF is also influenced by environmental conditions (Cheeke et al., 2019; Berger and Gutjahr, 2021). This is because symbiotic function of an AMF species is dependent on− and can vary with changes in soil chemistry. In this study, the term mycorrhizal species sensitivity has been used to refer to the variations in growth response of barley to *R. irregularis* under different fly ash concentrations in the growth substrate. Mycorrhizal responsiveness

and sensitivity of barley to *R. irregularis* under different fly ash concentrations were determined using the following equations:

$$\text{AMF responsiveness of barley} = \frac{\text{biomass of mycorrhizal plants}}{\text{biomass of non} - \text{mycorrhizal plants}}$$

The variances in AMF responsiveness were then calculated for plants grown under each fly ash concentration. Mycorrhizal sensitivity of barley to *R. irregularis* under different fly ash concentrations was measured by determining the coefficient of variation (CV) using the following equation (Cheeke et al., 2019):

$$\text{CV} = \frac{\text{variance in AMF responsiveness of barley}}{\text{average AMF responsiveness of barley}}$$

2.7. Nutrient and heavy metal analyses

Soil-fly ash substrate used in the microcosms and harvested plant tissues (separated into shoots, roots and grains) were acid digested with H_2SO_4–peroxide digestion (Allen, 1989). N (NO_3–N) and P (PO_4–P) content of soil-fly ash substrate and harvested plant tissues were determined using the indophenol blue (Cataldo et al., 1975) and molybdenum blue (Chen et al., 1956) methods, respectively. Ni, Co, Pb and Cr concentrations in soil-fly ash substrate and harvested plant tissues were measured using an Analytik Jena GmBH - novAA 350i (Germany) atomic absorption spectrophotometer (AAS).

2.8. Statistical analyses

Since the data were not normally distributed, differences in means were tested with a Kruskal-Wallis test followed by post hoc pairwise comparisons using Wilcoxon test with Bonferroni correction. A principal component analysis (PCA) was performed to explain the relationships among nutrient ions (N, P) and heavy metals (Ni, Co, Pb, Cr) concentrations in barley shoots, roots, grains and the growth substrate (after harvest) affected by mycorrhizal inoculation and fly ash treatments. A singular correlation matrix was computed, and all computations were based on the generalized inverse. Kruskal-Wallis tests and PCA were performed using Statistica (Version 12, StatSoft Inc.). Spearman rank correlations (ρ) were performed using R version 4.2.0. Significance was tested at $p \leq 0.05$.

3. Results

3.1. AMF colonization and plant biomass

Fly ash concentration in the substrate negatively impacted AMF colonization in roots of barley (arbuscles $\rho = -0.40$, $p = 0.02$; vesicles $\rho = -0.90$, $p < 0.0001$; hyphae $\rho = -0.40$, $p = 0.01$; $n = 40$). Percent AMF hyphal colonization of the root was significantly higher in plants grown with 15% fly ash compared to plants of the control (H (11) = 111.58, p =0.0006, n =120) and 30% fly ash amendments (H (11) = 111.58, p =0.02, n = 120) respectively (Fig. 1a). Percentage of vesicles was higher in the control compared to plants grown in soil with 30% fly ash amendment (H (11) = 111.58, p =0.03, n = 120). No differences were found in the percentage of arbuscles between plants of the control and those grown with 15 and 30% fly amendments respectively. No AMF colonization was detected in the non-mycorrhizal plants and the mycorrhizal plants grown with 50% fly ash amendment (Fig. 1a). Plant sensitivity to AMF colonization under different fly ash amendments, calculated as a coefficient of variation of the mycorrhizal responsiveness of barley, was highest at 30% and lowest in 50% fly ash amendments (Fig. 1b).

Mycorrhizal plants had higher shoot (H (7) = 73.22, $p < 0.0001$, n = 80) and root (H (7) = 69.76, $p < 0.0001$, n = 80) biomass than non-mycorrhizal plants at all concentrations of fly ash treatment. Shoot and root biomasses were highest in plants grown with 30% fly ash amendment (Fig. 2a and b). No significant difference was observed in root biomass of non-mycorrhizal plants grown in control and 15% (H (7) = 69.76, $p = 0.10$, $n = 80$) fly ash amendments respectively (Fig. 2b). Mycorrhizal plants of the control, 15 and 50% fly ash amendments had higher grain biomasses than their non-mycorrhizal counterparts (H (7) = 74.28; p = 0.0051, 0.0051 and 0.0050 respectively, n = 80). Plants grown with 30% fly ash amendment had the highest grain biomass (Fig. 2c). However, the difference was non-significant compared to its non-mycorrhizal counterpart (H (7) = 74.28, $p = 1.0$, $n = 80$); Fig. 2c).

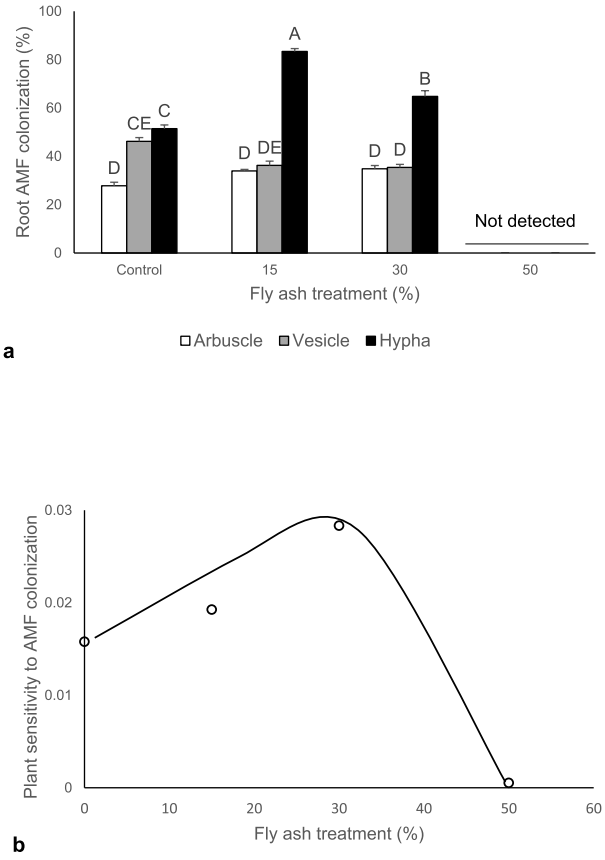

Fig. 1. Percent root colonization of *H. vulgare* by AMF *R. irregularis*. AMF colonization was not detected in 50% fly ash amendment. Values represent the mean of 10 replicates ± standard error of the mean. Values that do not share an alphabet are significantly different in a Wilcoxon pairwise comparison with Bonferroni correction, $p \leq 0.05$ (a). Mycorrhizal species sensitivity of *H. vulgare* to *R. irregularis* colonization when grown with fly ash amendments of 0 (control), 15, 30 and 50% respectively (b).

3.2. N and P uptake by H. vulgare plants

Mycorrhizal plants had higher shoot N-content than the non-mycorrhizal plants (H (7) = 76.75, $p < 0.0001$, n = 80). Shoot N was highest in mycorrhizal plants of the control. Mycorrhizal and non-mycorrhizal plants grown in 15% fly ash amendment had lowest shoot N. No difference in shoot N content was found between the mycorrhizal and non-mycorrhizal plants grown with 50% fly ash amendment (Fig. 3a).

N uptake in roots was highest in plants grown with 50% fly ash amendment (Fig. 3c). At 50% fly ash, no differences were found in root N of mycorrhizal and non-mycorrhizal plants (H (7) = 76.61, $p = 1.0$, n = 80). Mycorrhizal plants grown in the respective control, 15 and 30% fly ash amendments (H (7) = 76.61, p = 0.0051, 0.0050 and 0.0051, n = 80), had higher amounts of root N compared to the non-mycorrhizal

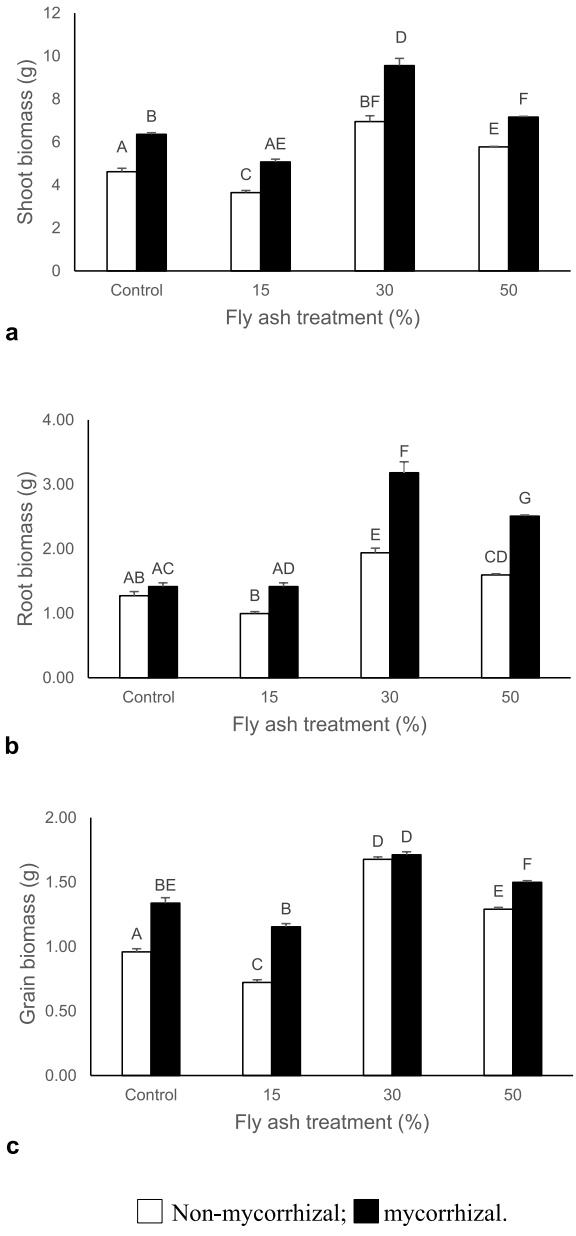

Fig. 2. Shoot (a); root (b); and grain (c) biomass of mycorrhizal and non-mycorrhizal *H. vulgare* grown with fly ash amendments of 0 (control), 15, 30 and 50% respectively. Values represent the mean of 10 replicates ± standard error of the mean. Values that do not share an alphabet are significantly different in a Wilcoxon pairwise comparison with Bonferroni correction, $p \leq 0.05$.

plants. Grains of mycorrhizal plants grown in the control (H (7) = 68.85, $p = 0.0003$, $n = 80$), 15% (H (7) = 68.85, $p = 0.005$, $n = 80$) and 30% (H (7) = 68.85, $p = 0.005$, $n = 80$) fly ash amendments had higher N content than those of the non-mycorrhizal plants (Fig. 3e). No differences in N content were found in grains of mycorrhizal and non-mycorrhizal plants grown with 50% fly ash amendment.

N localization was higher (H (23) = 234.92, $p < 0.0001$, $n = 240$) in the roots compared to shoots and grains of barley at all concentrations of fly ash amendment except the control (Supplementary S3a). In the control, N content was higher in the shoots compared to the roots of the mycorrhizal plants (H (23) = 234.92, $p = 0.05$, $n = 240$).

Mycorrhizal and non-mycorrhizal barley had higher P in roots than in the shoots and grains in all fly ash treatments (H (23) = 237.65; $p < 0.0001$, $n = 240$; Supplementary S3b). Shoots (H (7) = 77.10, $p = 0.004$, 0.005 and 0.004, $n = 80$) and roots (H (7) = 76.93, $p = 0.004$, 0.004 and 0.005, $n = 80$) of mycorrhizal plants had higher P, than those of non-mycorrhizal plants grown in the control, 15 and 30% fly ash amendment (Fig. 3b and d), respectively. Grains of mycorrhizal barley grown in the control, 15% and 30% fly ash amendments had higher P content than the non-mycorrhizal counterparts (H (7) = 76.90, $p = 0.0045$, 0.0048, 0.005, $n = 80$; Fig. 3f). No differences were found in P content of shoots, roots or grains of mycorrhizal and non-mycorrhizal plants grown at 50% fly ash amendment.

3.3. Heavy metal uptake by H. vulgare

Ni, Co, Pb and Cr concentrations in shoots, roots and grains were highest in plants grown in soils amended with 50% fly ash (Fig. 4a – d). Mycorrhizal and non-mycorrhizal plants grown in 50% fly ash treatment did not have significant differences in heavy metal content in shoots, roots or grains except for Co where at 50% fly ash concentration, uptake by the shoots (H (7) = 74.41, $p = 0.004$, $n = 80$) and roots (H (7) = 74.41, $p = 0.005$, $n = 80$) of the mycorrhizal plants respectively were higher than the non-mycorrhizal plants (Fig. 4b). Moreover, tissue heavy metal content was lowest in plants (mycorrhizal and non-mycorrhizal) grown in the control.

Shoots, roots and grains of mycorrhizal plants grown at 15 and 30% fly ash amendment had higher heavy metal content than their non-mycorrhizal counterparts (Fig. 4a – d). Concentrations of the heavy metals Ni (H (23) = 237.45, $p < 0.0001$, $n = 240$); Co (H (23) = 236.60, $p < 0.0001$, $n = 240$); Pb (H (23) = 236.60, $p < 0.0001$, $n = 240$) and Cr (H (23) = 237.49, $p < 0.0001$, $n = 240$) respectively, analysed in this study were highest in the roots and lowest in the grains (Supplementary S4a – d).

3.4. N, P and heavy metals residual in the growth substrate after harvest of barley

Post-harvest N content was lower in the substrate of non-mycorrhizal plants of the control compared to the mycorrhizal plants (H (7) = 76.14, $p = 0.0048$, $n = 80$). However, in substrates amended with 15 (H (7) = 76.14, $p = 0.005$, $n = 80$) and 30% fly ash (H (7) = 76.14, $p = 0.0051$, $n = 80$) respectively, post-harvest substrate N content of the mycorrhizal plants was lower than that of the non-mycorrhizal plants (Fig. 5a).

Substrate concentration of P, following harvest, was lower for mycorrhizal plants grown in the control, 15 and 30% fly ash amendments, respectively than the non-mycorrhizal plants (H (7) = 76.17, $p = 0.0048$, 0.0049 and 0.0049, $n = 80$; Fig. 5b). No differences were found in the post-harvest concentrations of N and P in the substrates of mycorrhizal and non-mycorrhizal plants grown with 50% fly ash amendment.

Post-harvest Ni (H (7) = 75.86, $p = 0.005$ and 0.004, $n = 80$), Pb (H (7) = 76.63, $p = 0.005$ and 0.0003, $n = 80$) and Co (H (7) = 69.36, $p = 0.0050$ and 0.0051, $n = 80$) concentrations in soils of mycorrhizal microcosms amended with 15 and 30% fly ash respectively were lower than in the non-mycorrhizal counterparts (Fig. 5c – e). Concentrations of Ni, Pb, and Cr, after the harvest of barley, were highest in soils amended with 50% fly ash. No differences were found in the post-harvest concentrations of Ni, Co, Pb or Cr for mycorrhizal and non-mycorrhizal plant grown in substrate amended with 50% fly ash concentration (Fig. 5c – f). In the control, substrates of the non-mycorrhizal plants had higher post-harvest concentrations of Ni (H (7) = 75.86, $p = 0.005$, $n = 80$), Pb (H (7) = 76.63, $p = 0.004$, $n = 80$) and Cr (H (7) = 76.06, $p = 0.007$, $n = 80$) respectively compared to substrates of the mycorrhizal plants.

In soils amended with 15% fly ash, residual concentration of Cr after the harvest of barley was lower in the non-mycorrhizal microcosms compared to the mycorrhizal microcosms (H (7) = 76.06, $p = 0.005$, $n = 80$).

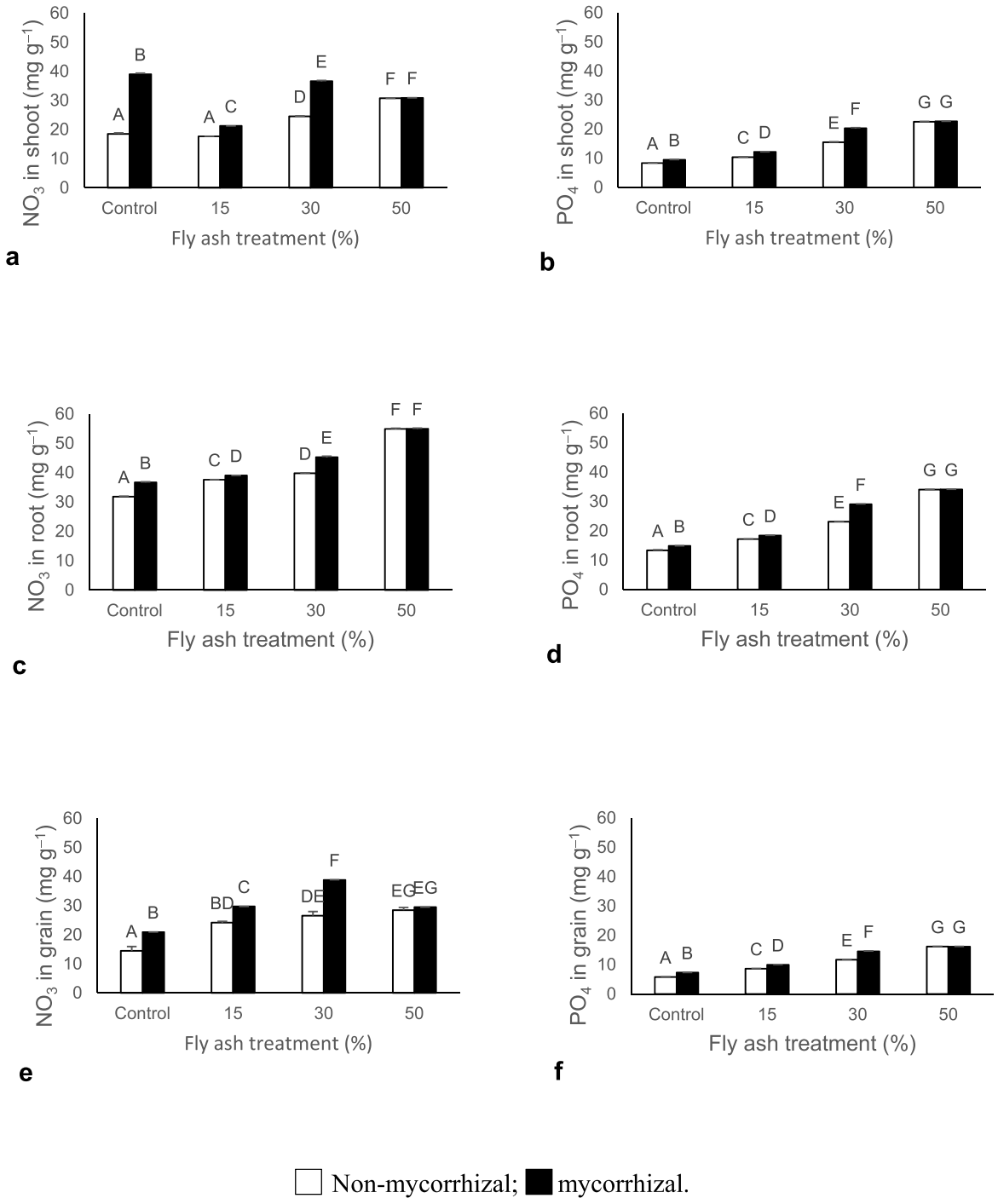

Fig. 3. NO_3–N and PO_4–P content in shoots (a, b), roots (c, d) and grains (e, f) of mycorrhizal and non-mycorrhizal *H. vulgare* grown with fly ash amendments of 0 (control), 15, 30 and 50% respectively. Values represent the mean of 10 replicates ± standard error of the mean. Values that do not share an alphabet are significantly different in a Wilcoxon pairwise comparison with Bonferroni correction, $p \leq 0.05$.

3.5. Principal component analysis

Principal component analysis showed that the first two principal component factors accounted for a cumulative 91.98% of the variance. Factors 1 and 2 accounted for respective 84.17 and 7.82% of the total variance (Table 1; Fig. 6). Concentrations of P, Ni, Pb and Cr in barley shoot, root and grain had strong correlation with fly ash treatment and *R. irregularis*. Co concentration in barley root correlated strongly with fly ash treatment and mycorrhizal inoculation (Table 1).

4. Discussion

Fly ash treatment and AMF colonization increased biomass, and N and P uptake of barley. Plant biomass is a function of N and P availability in soil (Yan, 2019). Fly ash amended soils had higher levels of N and P than the control (Supplementary S1). Shoots, roots and grains of *H. vulgare* plants grown in substrates with up to 30% fly ash amendment had higher N and P uptake when inoculated with *R. irregularis*.

The increase in plant biomass caused by a combination of fly ash amendments and inoculation with *R. irregularis* is an outcome of the increased nutrient uptake made possible by higher concentrations of N

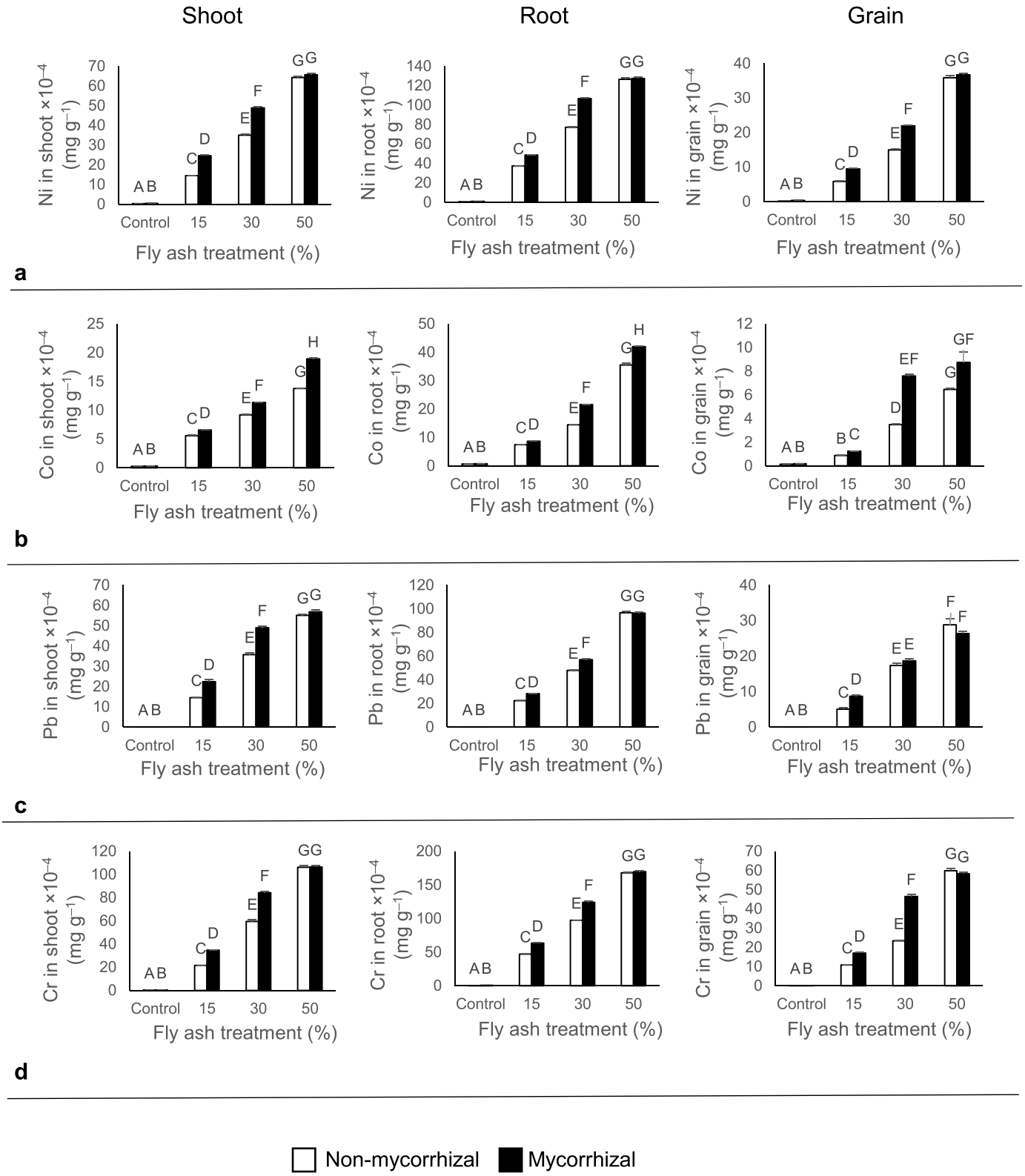

Fig. 4. Heavy metals Ni (a), Co (b), Pb (c), and Cr (d) concentrations in shoots, roots, and grains respectively of mycorrhizal and non-mycorrhizal *H. vulgare* following 16 weeks of growth with fly ash amendments of 0 (control), 15, 30 and 50% respectively. Values represent the mean of 10 replicates ± standard error of the mean. Values that do not share an alphabet are significantly different in a Wilcoxon pairwise comparison with Bonferroni correction, $p \leq 0.05$.

and P in fly ash, coalesced with similar arbuscular abundances in the roots of mycorrhizal plants of the respective control, 15 and 30% fly ash treatments (Fig. 1a; Fig. 3). Arbuscles are the sites of material exchange between the plant and the fungal symbiont (Luginbuehl and Oldroyd, 2017). Shoot, root and grain biomasses of mycorrhizal plants grown with 50% fly ash were higher than the non-mycorrhizal counterparts and also mycorrhizal plants grown at control and 15% fly ash amendment. This result is difficult to explain as AMF colonization was not

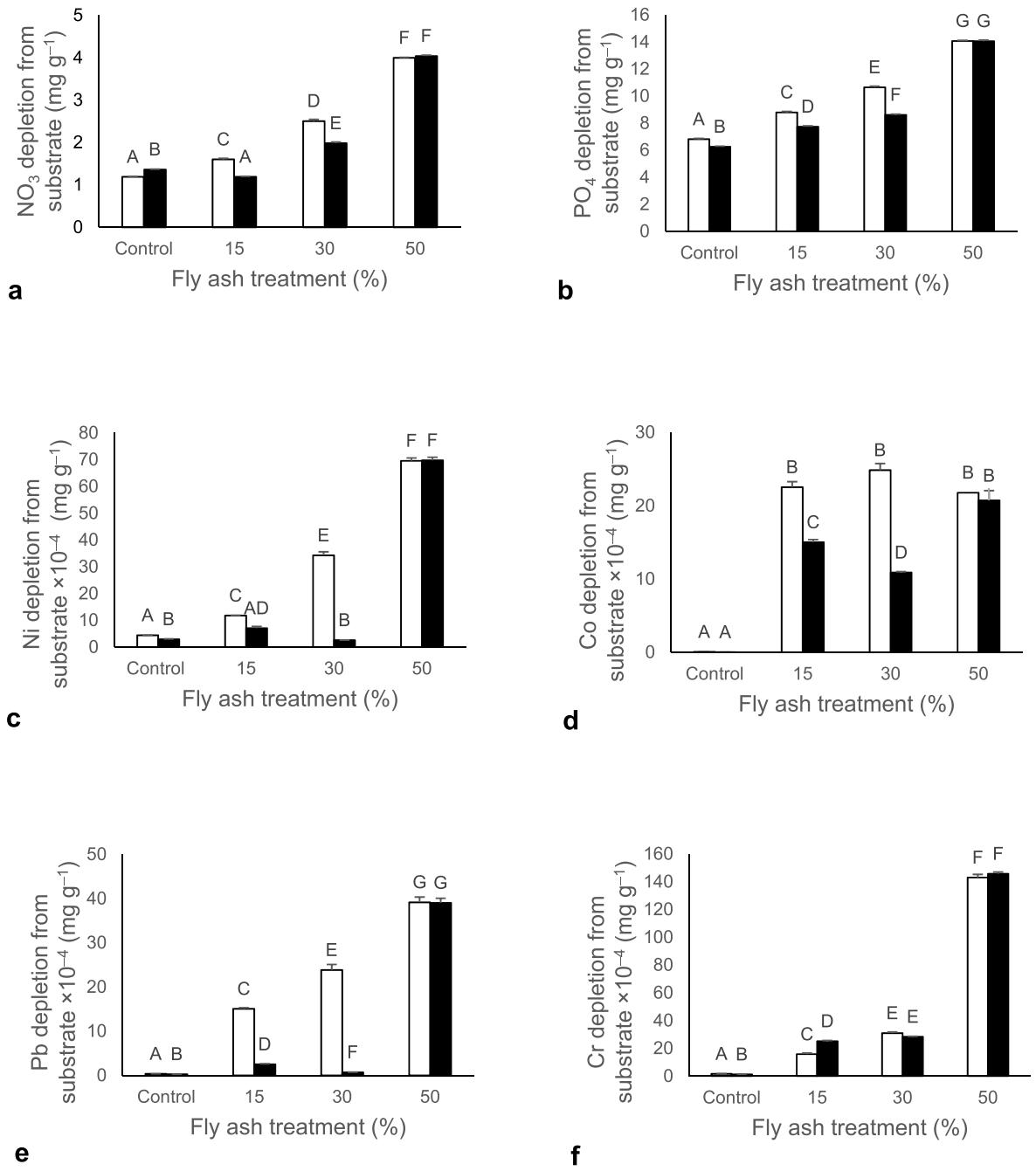

Fig. 5. NO_3–N (a) and PO_4–P (b) and heavy metals Ni (c), Co (d), Pb (e), Cr (f) remaining in the substrate after harvest of mycorrhizal and non-mycorrhizal *H. vulgare* following 16 weeks of growth with fly ash amendments of 0 (control), 15, 30 and 50% respectively. Values represent the mean of 10 replicates ± standard error of the mean. Values that do not share an alphabet are significantly different in a Wilcoxon pairwise comparison with Bonferroni correction, $p \leq 0.05$.

detected in roots of plants grown in mycorrhizal microcosms with 50% fly ash amendment (Fig. 1a). However, root colonization rates do not always represent the strength or function of mycorrhizal symbioses (Dietterich et al., 2017). No differences were found in N and P concentrations of mycorrhizal and non-mycorrhizal plants grown with 50% fly ash amendment. This was expected as no AMF colonization was detected in plants grown with 50% fly ash amendment.

Plant-AMF mutualisms are analogous to biological markets. Both share characteristics such as partner choice and adjustments to the demand and supply (Noë and Kiers, 2018). AMF impose costs of up to 20% of the photosynthetically fixed carbon on host plant in exchange for facilitating nutrient uptake (Smith and Read, 2008; Ray et al., 2020). Consequently, in nutrient poor soils, the benefits of acquiring nutrients through AMF symbioses outweigh the costs incurred in maintaining the AMF structures and favours cooperation (Kiers and van der Heijden, 2006). In nutrient-rich soils, such as the fly ash amended substrate used

Table 1
Eigen values and the principal component factor loadings for the correlation between nutrient and heavy metal content in shoots, roots, grains and growth substrate (after harvest) of barley affected by the interaction between fly ash concentration and inoculation with *R. irregularis*.

Principal Component	Factor 1	Factor 2
Eigen values	20.20	1.88
Variability (%)	84.17	7.82
Variables		
Root N	−0.97	−0.03
Shoot N	−0.34	−0.67
Grain N	−0.61	−0.60
Substrate N	−0.93	0.24
Root P	−0.99	−0.11
Shoot P	−0.98	−0.18
Grain P	−0.98	−0.17
Substrate P	−0.94	0.34
Root Ni	−0.98	−0.11
Shoot Ni	−0.99	−0.08
Grain Ni	−0.99	0.00
Substrate Ni	−0.86	0.44
Root Co	−0.99	0.03
Shoot Co	−0.97	0.00
Grain Co	−0.90	−0.27
Substrate Co	−0.64	0.39
Root Pb	−1.00	0.05
Shoot Pb	−0.97	−0.13
Grain Pb	−0.97	−0.01
Substrate Pb	−0.82	0.52
Root Cr	−0.99	−0.04
Shoot Cr	−0.99	−0.10
Grain Cr	−0.98	−0.13
Substrate Cr	−0.92	0.25

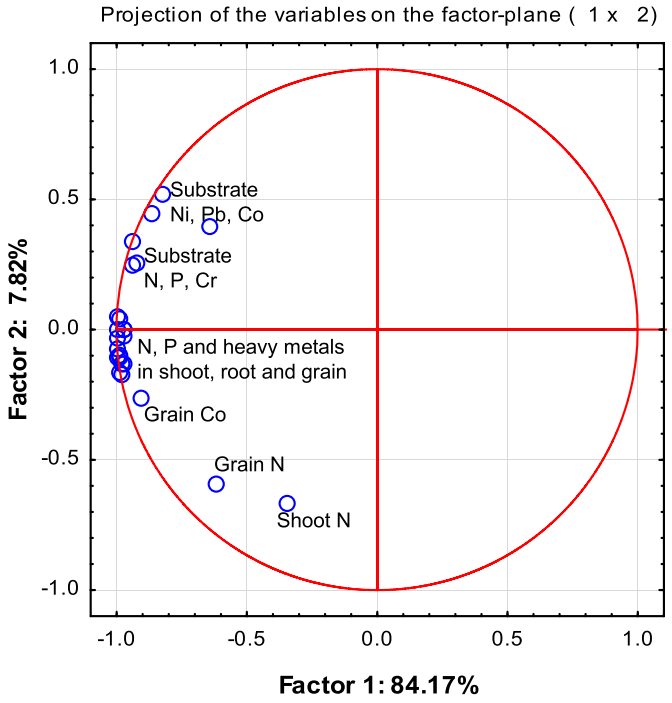

Fig. 6. Principal component projection of the correlation between nutrient and heavy metal content in shoots, roots, grains and growth substrate (after harvest) of barley affected by the interaction between fly ash concentration and inoculation with *R. irregularis*.

here, costs of maintaining AMF structures exceed the benefits from symbiosis (Johnson et al., 1997; Soka and Ritchie, 2015) and may cause a decrease in root AMF colonization. This is evident in the percentages of vesicles and hyphae in roots of plants grown in the control and with the fly ash treatments. Vesicles are carbon storage structures and the lower percentage of mycorrhizal vesicles in barley roots at 30% fly ash compared to the control and the absence of mycorrhizal structures at 50% fly ash may indicate reduced C translocation to the fungal symbiont (Titus et al., 2002). This could be a consequence of reduced reliance of the plants on AMF at higher concentrations of fly ash due to increased availability of N and P.

The higher hyphal percentage in roots of plants grown in 15 and 30% fly ash compared to the control is possibly a fallout of AMF investing a higher proportion of its resources in intraradical hyphae to improve storage of carbon extracted from the plants, especially when the fungi may have become carbon-limited due to reduced reliance of the plant on the symbiosis (Treseder et al., 2006). Moreover, AMF are sensitive to changes in the abiotic environment (Soka and Ritchie, 2014). Presence of pollutants like heavy metals may also influence root colonization by AMF. The significant negative correlation observed between fly ash concentration and mycorrhizal colonization in *H. vulgare* roots could be an outcome of higher N, P and heavy metal content in fly ash amended substrate (Supplementary S1). Studies analysing effects of metals on AMF colonization in roots have reported inconsistent results (Dietterich et al., 2017). In some studies, elevated metal concentrations decreased AMF colonization but increased in others (Khan, 2001; Vogel-Mikuš et al., 2006).

Despite the negative correlation of *R. irregularis* root colonization with fly ash concentration, sensitivity to AMF colonization was higher in barley grown in fly ash amendments of 15 and 30% compared to the control (Fig. 1b). Environmental conditions influence plant sensitivity to AMF colonization (Berger and Gutjahr, 2021). The decrease in biomass of barley grown in 50% fly ash compared to plants grown in 30% fly ash indicates a dose-dependent effect of fly ash on plant growth. Mitigation of adverse effects of fly ash on plant growth by AMF could be a reason for the higher mycorrhizal sensitivity of barley when grown in 15 and 30% fly ash amendments (Fig. 1b). Non detection of root AMF colonization and absence of mycorrhizal sensitivity at 50% fly ash treatment indicates that at this concentration, fly ash may also affect AMF metabolism.

The biomass benefits that fly ash amelioration in agricultural soils provides must be seen in the context of how they are outweighed by presence of toxic heavy metals like Ni, Pb, Co and Cr. In our study, both mycorrhizal and non-mycorrhizal plants had higher levels of Ni, Pb, Co and Cr than plants of the control (Fig. 4). The World Health Organization (WHO), Food and Agricultural Organization of the United Nations (FAO) and European Commission recommendations for permissible limits of Ni, Co, Pb and Cr in cereal grains are 1.0, 0.01, 0.2 and 1.0 μg g^{-1} respectively (World Health Organisation, 1989; 2008; Dhalaria et al., 2020). Our results showed that heavy metal content in grains of barley (mycorrhizal and non-mycorrhizal) grown in fly ash amended soil was higher than the permissible limits allowed for human consumption. Mycorrhizal colonization amplified heavy metal content in barley grains compared to the non-mycorrhizal treatments (Fig. 4). For instance, at 30% fly ash treatment, grains of mycorrhizal plants contained 46, 118, 7 and 99% higher amounts of Ni, Co, Pb and Cr, respectively than grains of the non-mycorrhizal plants. This must be seen in the context of similarity in percentages of arbuscles in mycorrhizal plants grown in the control, 15 and 30% fly ash amendments (Fig. 1a). Similar levels of arbuscular abundances but higher heavy metal concentrations in fly ash could be a reason for the amplified translocation of heavy metals to tissues of mycorrhizal plants compared to non-mycorrhizal plants (Fig. 4). Absence of differences in Ni, Pb and Cr levels in tissues of mycorrhizal and non-mycorrhizal plants grown with 50% fly ash may be attributable to the absence of intraradical AMF structures in mycorrhizal plants at this concentration. The relationship of fly ash concentration in soil and mycorrhizal inoculation with heavy metal uptake in barley is evidenced by the strong correlation of Ni, Pb and Cr concentrations in shoots, roots and grains of barley with fly ash treatment and mycorrhizal inoculation (Table 1; Fig. 6).

It is important to consider the consequences of fly ash amelioration in agriculture owing to the ubiquity of AMF in soils. *R. irregularis* used in this study could tolerate fly ash concentrations of up to 30% (≅ 275 t ha^{-1}; Fig. 1a). Moreover, presence of AMF in plants growing on fly ash dumps (Babu and Reddy, 2011) and the ability of *R. irregularis* to colonize *H. vulgare* in fly ash concentrations of up to 30% indicate that AMF propagules present naturally in agricultural soils will colonize crop roots even after amelioration with fly ash. Consequently, fly ash application to soils needs careful assessment. Once applied, the damage caused to soil due to accumulation of heavy metals and other pollutants may be irreversible (Taupedi and Ultra Jr, 2022). When viewed from a societal and human nutrition perspective, the increased uptake of heavy metals by mycorrhizal plants compared to non-mycorrhizal plants poses a heightened risk of their transfer to the food chain and humans. This is particularly problematic in the case of cereal crops like barley because heavy metals are localized in the grain endosperm (Watts-Williams and Gilbert, 2021), which is the edible part remaining after the milling process.

R. irregularis colonized barley were also extracting higher amounts of nutrients and heavy metals from the substrate than their non-mycorrhizal counterparts. This is evidenced by the lower residual concentrations of nutrients and heavy metals in the growth substrates of mycorrhizal barley following harvest (Fig. 5). It has been previously shown that AMF reduce leaching of nutrients from soils by retaining them in the fungal biomass (Martínez-Garcia et al., 2017; Goswami et al., 2023). This indicates that presence of AMF can have double edged consequences. They can transmit more heavy metals from the soil to the plant tissues and from thence to the food chain. But by extracting heavy metals at a faster rate than non-mycorrhizal plants, on a longer-term basis they remove them from the soils. Moreover, significantly higher amounts of Ni, Co, Pb and Cr were localized in the roots of barley rather than in shoots or grains (Supplementary S4). Shoots and grains are the primary modes of transfer to the food chain. Roots of mycorrhizal barley contained higher amounts of heavy metals than the non-mycorrhizal plants (Fig. 4). Plants have evolved heavy metal exclusion mechanisms that serve as barriers to their movement from root to shoot (Yan et al., 2020). AMF amplify retention of heavy metals in the roots by increasing the absorptive surface area of the roots, retaining heavy metals in mycorrhizal structures such as the fungal mycelium and vesicles, and preventing heavy metal mobilization to aerial plant tissues (Dhalaria et al., 2020). Nevertheless, the fate of mycorrhiza-mediated movement of heavy metals to the food chain needs more investigation.

5. Conclusions

Our results show that fly ash amendments improve crop yield through increased grain biomass. But the increased yield and resultant economic profits from use of fly ash as a soil amendment may come with heightened health risks. Our microcosm-based experiment provides evidence that AMF in the soil indeed amplify the risk of heavy metal transfer to the human food chain from fly ash added to soil through increased transfer to edible tissues of barley. However, our results indicate that fly ash amelioration at 50% concentration inhibits AMF colonization in the roots. The inhibitory effects of fly ash on AMF colonization need further investigation. An important limitation of our study is that the experiment is microcosm-based with controlled conditions. Long-term field-based studies analysing AMF responses to fly ash are needed to understand the effects of chronic exposure to fly ash amelioration on rhizospheric AMF communities and the human health risks associated with AMF mediated amplification of heavy metal uptake in agricultural crops.

Author statement

Vikrant Goswami: Pot experiments, sample analyses. Sharma Deepika: Pilot experiment, writing. Swati Diwakar: Statistical analyses, writing. David Kothamasi: Conceptualization, supervision, pilot experiment, statistical analyses, writing.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.121733.

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