Citric acid and AMF Inoculation Combination Assisted phytoextraction of vanadium (V) by Medicago Sativa in V Mining Contaminated Soil

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Abstract

The use of citric acid (CA) chelator to facilitate metal bioavailability is a promising approach for phytoextraction of heavy metal contaminants. However, the role of CA chelator associated with arbuscular mycorrhizal fungi (AMF) inoculation on phytoextraction of vanadium (V) has not been studied. Therefore, in this study, a greenhouse pot experiment was conducted to evaluate the combined effect of CA chelator and AMF inoculation on plant growth and V phytoextraction in the V mining contaminated soil by *Medicago sativa* Linn. (*M. sativa*). The experiment was performed via CA (at 0, 5 and 10 mM kg^{-1} soil levels) application alone or in combination with AMF inoculation. Plant biomass, root mycorrhizal colonization, P and V accumulation, antioxidant enzyme activity in plant, and soil chemical speciation of V were evaluated. Results depicted (1) a marked decline in plant biomass and root mycorrhizal colonization in 5- and 10-mM CA treatments which were accompanied by a significant increased V accumulation in *M. sativa* tissues. The effects could be attributed to the enhancement of bioavailable V by mainly transferring from the reducible to acid-soluble V fraction. (2) The presence of CA significantly enhanced P acquisition while the ratio of P/V concentration in plant shoots and roots decreased, owing to the increased V translocation from soil to plant. (3) In both CA treated soil, AMF symbiosis significantly improved dry weight (31.4–73.3%) and P content (37.3-122.5%) in shoot and root of *M. sativa*, and showed markedly contribution in reduction of malondialdehyde (MDA) content (12.8–16.2%) and higher antioxidants (SOD, POD and CAT) activities in the leaves, suggesting their combination could promote growth performance and stimulate antioxidant response alleviating V stress induced by CA chelator. (4) Taken together, 10 mM kg^{-1} CA application and AMF inoculation combination exhibited higher amount of extracted V both in the shoot and root. Thus, citric acid-AMF-plant symbiosis provides a novel remediation strategy for *in situ* V phytoextraction by *M. sativa* in the contaminated soil.

1 Introduction

Vanadium (V) is a transition metallic element and widely distributed in the lithosphere (Hao et al., 2018). Generally, V concentration in Earth's crust is 150 mg kg^{-1}, but varied with soil types in the range of 2-310 mg kg^{-1} (Rehder, 1991). As a valuable strategic resource, it is widely used in machinery manufacturing, aerospace, railways and other fields (Moskalyk and Alfantazi, 2003; Schlesinger et al., 2017). High concentration in soil and water environments can lead to detrimental effect on plant growth and all living organisms, although it in trace amounts is an essential for human beings and animals (Crans et al., 2004; Yang et al., 2017). Thus, the United Nations Environment Programme (UNEP) has put vanadium on the priority list of environmental hazardous elements in 1980s (Hindy, 1990). In China, about 53% of vanadium minerals have been produced from varieties of vanadium-titanium magnetite mines and account for the current global output (Chen et al., 2020), but the mining and smelting activities have resulted in surrounding soil enriched with V pollution, especially in Panzhihua and Huaihua city where vanadium concentration in soil severely exceeds the background value in China (82 mg kg^{-1}) (Xiao et al., 2015; Zhang et al., 2019). Additionally, the characteristics of nutrient deficiency, extreme pH, and decreased microbial diversity, accompanied in V mining contaminated soil, consequently cause an

impoverished habitat hindering plant establishment (Xiao et al., 2015). Hence, it is urgent to develop sustainably and economically efficient techniques for remediation of vanadium polluted sites.

Phytoextraction, as a subgroup of phytoremediation, can utilize specific plants to enrich heavy metals in the aerial parts to remove metals in soil (Freitas et al., 2013). This technology can be used in large area of mine reclamation and heavy metal contaminated sites owing to its cost-effectiveness, no secondary damage and high efficiency (Sarwar et al., 2017). For a better performance, metal bioavailability in soil is a key factor in controlling the success of phytoextraction. Based on BCR (Community Bureau of Reference) fractionation, soil heavy metals can be categorized into different forms including acid-soluble, reducible, oxidizable and residual fraction (Hao et al., 2018). Among them, soil acid-soluble fraction represents the most mobile fraction that can be absorbed by plants. However, V element exist mainly in residual fraction in soil (Xiao et al., 2015; Hao et al., 2018), and the low metal bioavailability strongly weakens phytoextraction efficiency (Wang et al., 2019).

In recent years, many studies have focused on the effects of chelator on controlling the solubility of metals in soil such as EDTA (ethylenediaminetetraacetic acid) and some low molecular organic acids (LMWOA) including citric acid, oxalic acid or malic acid, those of which effectively enhance metal mobility and diffusion to root surface, thereby boosting phytoextraction (Blaylock et al., 1997; Sinhal et al., 2010; Freitas et al., 2013; Farid et al., 2017; Wang et al., 2019). Importantly, citric acid (CA), as a natural chelating agent, has been reported a better substitute to synthetic chemical chelator for phytoextraction because of its low cost and effortless degradation without leaching metal-chelator compounds (Farid et al., 2017), and currently numerous studies associated with CA on phytoextraction of Cr (Farid et al., 2017), Cu (Zaheer et al., 2015), Cd (Sinhal et al., 2010), Pb (Shakoor et al., 2014) and As (Almaroai et al., 2012) for decontaminating polluted soils have been reported intensively, but less attention has been paid on V phytoextraction. However, higher concentration of CA and/or elevated bioavailable metals in phytoremediation also result in severe phytotoxicity symptoms such as plant growth inhibition (Turgut et al., 2004; Yang et al., 2017), interfering with nutrients uptake (Cao et al., 2009) and inducing overgeneration of reactive oxygen species (ROS) (Imtiaz et al., 2015). Higher induced ROS production could further cause oxidative damage and retard plant growth by disturbing physiological and biochemical activities.

Arbuscular mycorrhizal fungi (AMF), which form mutualistic symbioses with most terrestrial plants, can improve plant tolerance against both biotic and abiotic stresses such as that from heavy metal (Dhawi et al., 2016). AMF symbiosis can alleviate oxidative damage by increasing the activity of antioxidants such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) to scavenge the ROS (Mollavali et al., 2016). Furthermore, some studies have reported the mechanisms of AMF symbiosis in mediating metal transportation and accumulation in soil-plant system for boosting metal tolerance (Chen et al., 2004). Thus, AMF-plant symbiosis has been treated as the potential candidate for phytoremediation (Miransari, 2011; Ma et al., 2019; Wang et al., 2019). In addition, AMF symbiosis take an active part in improved absorption of mineral nutrition (particularly P), and this improvement of plant P nutrition associated with AMF symbiosis has been generally regarded as plant tolerance mechanism against

heavy metals' toxic effect (Wu et al., 2016). However, the role of AMF symbiosis in V accumulation and physiological responses against vanadium stress has not been studied intensively. Moreover, owing to the similar structure between vanadate ($H_2VO_4^-$) and phosphate ($H_2PO_4^-$) ions (Rehder 2015), plant phosphorus status in phytoremediation may be interactive with plant V accumulation induced by the individual effect of CA application or AMF inoculation, or their combined effect.

Medicago sativa Linn. (*M. sativa*), as a perennial legume herb plant, has a characteristic of high biomass and well-developed root system, and has been reported to be tolerant and ecologically adapt to heavy metal stress (Yang et al., 2011; Gan et al., 2020). Typically, *M. sativa* can be cultivated on varying types of soil for potential phytoremediation of heavy metals such as Cd, Pb, Cr, Ni, Zn and V (Peralta-Videa et al., 2002; Yang et al., 2011; Zhang et al., 2020). It is widely distributed and easily propagated in China and around the world, and can be used in vegetative restoration or a source of livestock feed (Hou et al., 2020). Therefore, *M. sativa* could be used as a positive candidate for phytoextraction to targe V from the contaminated soil. However, to the best of our knowledge, there are currently few reports on the effects of CA application and AMF inoculation on *M. sativa*'s growth performance and V accumulation.

Therefore, this study aimed to evaluate the combined effects of CA application and AMF inoculation on V phytoextraction by *M. sativa* grown in vanadium contaminated soil by measuring the growth, root mycorrhizal colonization, P uptake, V accumulation and antioxidant enzymes activity, Malondialdehyde (MDA) production, and soil chemical speciation of V in rhizosphere with either CA application, AMF inoculation, or their combination.

2 Materials And Methods

2.1 Cultivation media, AMF inoculant and seeding

In September 2018, soil samples (0–20 cm) were collected in the vicinity of vanadium mine spoil (27°59'54"N, 110°41'15"E), at Chenxi Country, Hunan Province, south China. Soil physiochemical properties were presented in Table 1. After removing coarse or fine root, and other visible impurities, the 2-mm-mesh sieved soils were autoclaved at 121℃ for 2 h to get rid of living AMF spores, and then air-dried for potting. The AMF inoculum, *Funneliformis mosseae* BGC XJ01, was adopted in this study. The initial inoculum was purchased from Beijing Academy of Agriculture and Forestry Science, and then cultivated in sterile sand with maize for 3 months (Bi et al., 2018). The AMF inoculum used consisted of infected root segments (90% mycorrhizal root colonization), external mycelium and fungal spores (1610 spores per 100 g soil). Seeds of *M. sativa* were surface-sterilized with 10% H_2O_2 for 10 min, then rinsed with sterile water several times. Sterilized seeds were germinated on moist filter paper in dark condition for several days till sprouting then for further pot experiment.

Table 1
Soil chemical properties, texture and total metal concentration of the study site, Chenxi Country, Hunan Province, China.

Soil chemical properties	
pH (H_2O)	6.91
SOC (g kg^{-1})	2.30
Total N (g kg^{-1})	0.42
Total P (g kg^{-1})	0.71
Total K (g kg^{-1})	6.62
Available N (mg kg^{-1})	54.6
Olsen-P (mg kg^{-1})	5.63
Available K (mg kg^{-1})	61.2
Soil texture (%)	
Sand (0.05-2 mm)	53
Silt (0.002–0.05 mm)	25
Clay (< 0.002 mm)	22
Metal concentration in soil (mg kg^{-1})	
V_{total}	1705
$V_{acid-soluble}$	94.4
$V_{reducible}$	389.2
$V_{oxidizable}$	295.8
$V_{residual}$	925.6
Cr	169.7
Cd	2.09
Cu	77.8
Pb	100.5
As	124.4

2.2 Experimental design

Before pot experiment, a gradient experiment was conducted to determine the optimum CA dosage to make chelator-vanadium soluble complexes in the soil solution. Briefly, 4 g dried soil were added to 40 mL of citric acid solution with different concentration gradients (0, 2.5, 5, 10, 15, 20, 25 mM·kg^{-1} soil) in a 150 mL volume of Erlenmeyer flask, and shaken at 25°C for 16 h on a reciprocal shaker (Crystal IS-RDV1), then centrifuged at 4000 r min^{-1} for 10 min. The 0.45 μm-membrane filtrated extract was determined with ICP-MS (Perkin-Elmer, NexION 300, USA). Each treatment repeated 5 times. Citric acid solution was prepared from analytical reagent (≥ 99.5%, Macklin). Results showed 5- and 10-mM kg^{-1} CA concentration was suitable for the release of chemically extractable V from the contaminated soil (Fig. S1 in the Supporting Information).

The pot experiment adopted a 2×3 factorial with AMF inoculation and CA application in a completely randomized design. Six treatments with four biological replicates were set up, including T1: control designated as CA0-M, T2: non-AMF inoculation and CA (5 mM kg^{-1}) designated as CA5-M, T3: non-AMF inoculation and CA (10 mM kg^{-1}) designated as CA10-M, T4: AMF inoculation and without CA designated as CA0 + M, T5: AMF inoculation and CA (5 mM kg^{-1}) designated as CA5 + M, and T6: AMF inoculation and CA (10 mM kg^{-1}) designated as CA10 + M. The control plants were treated with non-AMF inoculation and without CA application.

Dried soil (1.1 kg) was put into each cleaned plastic pot (height 10.5 cm, upper diameter 14.3 cm and bottom diameter 9 cm), then saturated with deionized water to keep soil moisture balance for two weeks. For AMF inoculation treatments, 50 g AMF inoculum were added into the upper soil of pot, then mixed and covered with about 100 g soil. The corresponding un-inoculated treatments were the same operation with equal weight of sterilized inoculum. After 60 d of cultivation, the CA solution was applied with watering to the corresponding CA application treatments by twice at one-week interval. Two dose of CA solution has no toxic symptoms to plant growth, and plants were harvested at 90 d of cultivation. Each pot was fertilized with 100, 20 and 100 mg kg^{-1} N, P and K, respectively, as controlled by addition of KNH_4NO_3, KH_2PO_4 and KNO_3. 50 *Medicago sativa* seeds were sown in each pot, and then thinned to 15 uniform-sized plants after germination. All pots were watered every two day in order to keep soil moisture at 60% of maximum water holding capacity and changed positions irregularly. Pots were placed in the open greenhouse from April to June of 2020 with natural lighting at temperature of 20–30°C throughout plant growth.

2.3 Plant and soil sampling

At destructive harvest, plant shoots and roots were separated and carefully washed. Before processing, a handful of fresh plant leaves and roots were collected and washed with deionized water for the determination of antioxidant enzyme activities and root mycorrhizal colonization, respectively. The loose soil attached to the individual plant root was shaken off, and collected and mixed uniformly to form a rhizosphere soil sample. Then, the remaining plant samples were firstly deactivated at 105°C for 30 min,

and then dried at 70℃ in the oven to the constant weight and weighed for plant biomass. Soil samples were sieved with a 2-mm mesh to remove coarse or fine root, and other visible impurities, and then air-dried and used for measurement of soil chemical properties.

2.4 Measurement of mycorrhizal colonization, P and V concentration in plant

Mycorrhizal root colonization was determined by the method of Phillips and Hayman (1970). Briefly, the washed roots were sufficiently softened with 10% KOH for 24 h, and then stained with 0.05% trypan (w/v) in lactic acid, glycerol and water solution (1:1:1 v/v/v) for 12 h after rinsing in water, and then decolorized with 50% acid glycerol solution. We randomly selected two groups of 15 root segments (1.5-cm-long) for individual plant root, and then deposited them on the slides for microscopy. The percentage of root mycorrhizal colonization (%) was calculated by the grid-line intersect method (Giovannetti and Mosse,1980).

For plant P and metal analysis, 0.2 g milled plant samples of shoot (stem and leaves) and root were respectively digested in a mixture of concentrated HNO_3/HCl (6:2 v/v) on the microwave system (Mars5, CEM Corporation, USA). Then, the wet digestion samples were filtered and diluted with 1% HNO_3, and analyzed for P and V in plant samples by ICP-MS. V accumulation amount (mg pot^{-1}) in plant was measured by multiplying V concentration in shoot or root organ (mg kg^{-1}) with the corresponding organ dry weight (kg pot^{-1}). Additionally, the bioaccumulation factor (BCF), translocation factor (TF) and metal extraction amount were introduced to evaluate phytoextraction efficiency of V in plant shoot and root. BCF was calculated as the ratio of metal concentration of plant shoot to the initial soil. TF was determined by the metal concentration of shoot divided by that of root.

2.5 Evaluation of physiological indices

Malondialdehyde (MDA) content in plant leaves was determined with minor modified method of thiobarbituric acid (TBA) reaction by Heath and Packer (1968). Briefly, 0.2 g of shoot sample was added with 2 mL of 10% trichloro acetic acid (TCA) to grind into tissue homogenate. The homogenization was centrifuged for 10 min at 12,000×g. Then, the assay solution was mixed by addition of centrifuged supernatant to an equal volume of 0.67% TBA. The resultant mixture was suddenly cooled after boiling in water bath for 30 min. After centrifugation for 10 min at 12,000×g, the obtained supernatant was determined spectrophotometrically followed by absorbance at 450, 532 and 600 nm wavelength, and ddH_2O was used as the blank.

Antioxidant enzymes including superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6) in the leaf samples were evaluated in this study. Known weights of fresh leave samples were firstly frozen in liquid nitrogen, and put into a pre-cooled mortar with 2.0 mL of 50 mM phosphate buffer (pH 7.8). The mixture was grinded into homogenate on ice bath, and then centrifugated for 20 min at 4℃ and 12, 000×g, and the supernatant was collected in tube for antioxidant enzymes assays.

SOD activity was measured by Total Superoxide Dismutase (T-SOD) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. One unit of SOD was defined as the amount of SOD when inhibiting the reduction of SOD by 50% per gram fresh tissue in 1 mL reacted solution, as monitored at 550 nm with a UV-1600 spectrophotometer (Zhang et al., 2017). POD activity was detected according to Maehly and Chance (1954). The assay mixture was comprised of 30 μL enzyme extract, 3 mL 0.2 M phosphate buffer (pH 6.0), 76 μL guaiacol and 0.112 mL H_2O_2 (30%). One unit of POD was defined as the increase in absorbance by 0.01 per minute and per gram fresh tissue at 470 nm. CAT activity was determined by the method of Knörzer et al. (1996). The assay mixture contained 100 μL enzyme extract, 3 mL 0.15 M phosphate buffer (pH 7.0) and 0.3092 mL H_2O_2 (30%). One unit of CAT was defined as the decrease in absorbance by 0.01 per minute and per gram fresh tissue, as monitored at 240 nm.

2.6 Determination of soil chemical speciation of vanadium and pH

Soil chemical speciation of vanadium (acid-soluble, reducible, oxidizable and residual) was analyzed according to the Community Bureau of Reference (BCR) sequential extraction procedure with some amendments (Hao et al., 2018). The detailed procedures of modified BCR sequential extraction were described in Table 2. In addition, V concentration of the residual fraction and total in soil samples were digested with an acid mixture (HNO_3/HCl/HF; 6:3:1 v/v/v) through the microwave system. Digestion solution was transferred into polytetrafluoroethylene (PTFE) tubes to evaporate nearly dry, and then dissolved and diluted to 50 mL with 1% HNO_3. Both total and sequential extracted V filtrates were determined with ICP-MS. The certified standard reference materials (GBW 07453) obtained from the China National Center for Standard Reference Materials was measured for metal concentration by 86.3%-98.2%. Soil pH was measured in a 1:2.5 (m/v) ratio of soil: water by pH meter (Sartorius, PB-10).

Table 2
The details of modified Community Bureau of Reference (BCR) extraction procedure.

Chemical speciation	Extraction procedures	Extraction conditions	ratio of soil to solvent
Acid-soluble fraction	HOAc (0.11M)	Oscillation 150 rpm for 16 h at 25°C	1:40
Reducible fraction	NH_2OH·HCl (0.5 M, pH 1.5)	Oscillation 150 rpm for 16 h at 25°C	1:40
Oxidizable fraction	H_2O_2 (30%, pH 2–3), NH_4Ac (1.0 M, pH 2)	Intermittent oscillation 120 rpm in water bath (85°C) for 1 h; repeated the operation with 10 mL H_2O_2	1:50
Residual fraction	HNO_3/HCl/HF (6:3:1 v/v/v)	Microwave digestion for 2 h	/

2.7 Statistical analysis

All the data was presented as means of four replicates, and complied with normal distribution and homogeneity prior to conducting significance analyses. Two-way ANOVA was conducted to analyze the impact of CA application and AMF inoculation on each variable studied using SAS 8.02 software (SAS Institute, Cary, NC). Least Significant Difference (LSD) test was used to determine significant differences between treatments at the levels of 5% when ANOVA was significant. Pearson's correlation analysis was used to evaluate the relationship between soil pH and acid-soluble V, plant biomass and P content in shoot, plant biomass and P content in root, shoot biomass and root mycorrhizal colonization, root biomass and root mycorrhizal colonization. Graph plotting were performed with SigmaPlot version 14.0.

3 Results

3.1 Mycorrhizal colonization and plant biomass

In this study, mycorrhizal colonization in *M. sativa* root ranged from 49.2 to 60.8% throughout all samples. These high colonization rates demonstrate the symbiosis between *Funneliformis mosseae* and host plant was successfully established (Fig. 1a). In all CA treatments, presence of AMF symbiosis brought by the successful colonization significantly promoted the dry weight of both plant shoot and root by 31.4–43.5% and 63.2–73.3%, respectively, when compared to associated sole CA application treatments. Though low colonization rates (0.83%) in plant root were observed in the control and 10 mM sole CA treatments, these low rates were probably due to the indigenous mycorrhizal fungi infection from the slightly incomplete sterilized soil.

However, addition of CA significantly decreased root mycorrhizal colonization rate as compared to those respective AMF-inoculated treatments without CA addition (Fig. 1b). Similarly, addition of CA treatment significantly reduced dry weight of plant shoot (17.0-27.4%) and root (26.2–37.1%), regardless of the existence of AMF inoculation. In addition, this decrease on plant dry weight was associated with increased CA concentration, suggesting CA chelator at concentration of 5- and 10-mM kg^{-1} soil showed some inhibitory effect on plant growth.

3.2 Phosphorus concentration in samples

In Fig. 2a, it displays P concentrations in both shoot and root of *M. sativa* that were cultivated in the vanadium contaminated soil gradually increased with increasing CA addition levels. Compared with the control plants, P concentrations in shoot and root were significantly increased in both 5- and 10-mM CA treatment (43.78–67.9% and 18.9–48.9%, respectively) (Fig. 2a), suggesting CA application would facilitate P transportation and accumulation from soil to plant tissue. Furthermore, AMF inoculated plants presented higher concentration of P both in shoot and root as compared to respective treatments without AMF inoculation. This is possibly as a consequence of AMF promotion on P absorption in plant.

In root, the P concentrations were significantly influenced by the interaction of AMF inoculation and CA application, according to the two-way ANOVA analysis (Table 3). In shoot, AMF inoculation gradually increased P content (mg per pot) in shoot with increasing levels of CA addition when plants were exposed to 5- and 10-mM CA relative to control plants (Fig. 2b). The highest P content in shoot was achieved in the group with combined treatment of 10 mM CA and AMF inoculation.

Table 3

F-values and significance of two-way ANOVA of the impact of AMF inoculation and citric acid application on growth, nutrient uptake, metal accumulation, and physiological indices of *M. sativa* and rhizosphere soil chemical speciation of vanadium ($n = 24$).

Items	AMF	Citric acid	AMF × Citric acid
Mycorrhizal colonization (%)	813.44^{***}	4.0^{*}	3.25^{NS}
Shoot dry weight (g pot^{-1})	25.39^{***}	7.80^{**}	0.12^{NS}
root dry weight (g pot^{-1})	26.86^{***}	10.75^{**}	0.14^{NS}
P concentration in shoot (mg kg^{-1})	44.26^{***}	48.83^{***}	0.93^{NS}
P concentration in root (mg kg^{-1})	112.01^{***}	22.84^{***}	5.47^{*}
P content in shoot (mg pot^{-1})	42.04^{***}	2.95^{NS}	1.06^{NS}
P content in root (mg pot^{-1})	31.69^{***}	1.11^{NS}	0.46^{NS}
V concentration in shoot (mg kg^{-1})	1.22^{NS}	350.58^{***}	0.89^{NS}
V concentration in root (mg kg^{-1})	22.05^{***}	92.69^{***}	7.32^{**}
Ratio of P/V concentration in shoot	0.42^{NS}	64.61^{***}	0.53^{NS}
Ratio of P/V concentration in root	18.91^{***}	17.01^{***}	0.90^{NS}
BCF	0.23^{NS}	1.61^{NS}	4.70^{*}
TF	0.10^{NS}	0.96^{NS}	2.02^{NS}
V extraction amount in shoot (mg pot^{-1})	7.47^{*}	111.99^{***}	2.01^{NS}
V extraction amount in root (mg pot^{-1})	7.07^{*}	30.39^{***}	0.28^{NS}
MDA (umol g^{-1} FW)	0.31^{NS}	7.16^{**}	21.94^{***}
SOD (U g^{-1} FW)	0.34^{NS}	1.50^{NS}	1.06^{NS}
POD (U g^{-1} min^{-1} FW)	0.08^{NS}	0.99^{NS}	2.01^{NS}
CAT (U g^{-1} min^{-1} FW)	0.05^{NS}	0.29^{NS}	1.67^{NS}
V_{total} (mg kg^{-1})	6.55^{*}	70.72^{***}	0.17^{NS}

BCF: bioaccumulation factor, TF: translocation factor, MDA: malondialdehyde, SOD: superoxide dismutase, POD: peroxidase, CAT: catalase; NS, *, ** and *** indicates not significant, significant difference at 5%, 1% and 0.1% level, respectively.

Items	AMF	Citric acid	AMF × Citric acid
$V_{acid-soluble}$ (mg kg^{-1})	6.8^{*}	39.4^{***}	3.51^{*}
$V_{reducible}$ (mg kg^{-1})	16.38^{***}	124.61^{***}	2.83^{NS}
$V_{oxidizable}$ (mg kg^{-1})	1.12^{NS}	22.49^{***}	0.41^{NS}
$V_{residual}$ (mg kg^{-1})	1.65^{NS}	4.74^{*}	5.71^{*}
pH (H_2O)	0.25^{NS}	34.07^{***}	0.04^{NS}

BCF: bioaccumulation factor, TF: translocation factor, MDA: malondialdehyde, SOD: superoxide dismutase, POD: peroxidase, CAT: catalase; NS, *, ** and *** indicates not significant, significant difference at 5%, 1% and 0.1% level, respectively.

3.3 Vanadium concentration and ratio of P/V concentration in plant

V concentrations in shoot were only 12 mg kg^{-1} in control and 22 mg kg^{-1} in single AMF inoculation treatment, resulting in a 44.2–74.8 times higher values in root than these in shoot. This indicates that *M. sativa* accumulated a higher amount of V in root than in shoot (Fig. 3). Application of CA significantly improved V concentration in both shoots and roots of *M. sativa* compared to plants without CA addition. Both maximum V concentrations in plant shoot and root samples were achieved in single 10 mM kg^{-1} CA application treatment. AMF inoculation showed no significant impact on V concentration in plant shoot under three levels of CA application compared with their respective CA treatment (Table 3; Fig. 3), while in root samples, V concentration was significantly reduced by 35.4% and 31.2% when plants were treated with AMF inoculation along with 5- and 10-mM CA application, as compared to the respective CA only treatment. This result explicitly presents that AMF symbiosis could suppress V absorption when the bioavailable V in soil was increased by CA chelator.

Concerning the P/V ratio in plant issues, both CA addition treatments (5 and 10 mM) significantly decreased the ratio in both shoot and root of *M. sativa*, as compared to non-CA treated plants (Fig. 4). When inoculated with AMF, P/V concentration ratio in shoot was enhanced by 29.7% and 47.1% under 5- and 10-mM CA treatments, respectively, as compared to the respective CA-only treated plants (Fig. 4a). Similarly, in plant root, AMF inoculation exhibited significant positive effect on the P/V concentration ratio regardless of CA concentrations (Table 3; Fig. 4b).

3.4 BCF, TF and V extraction amount by plant from soil

As shown in Table 4, higher BCF values (0.50–0.83) of *M. sativa* were obtained in both CA application treatments. Similarly, TF values were also significantly higher in both single CA treated plants than the non-CA treated plants, indicating that CA application could enhance the ability to transport and accumulate V by *M. sativa* in the contaminated soil. Moreover, when considering the combined effect of

both CA and AMF inoculation, the combined treatments significantly enhanced BF values as compared to respective treatments with CA application alone. Maximum V extraction amount in plant shoot and root were obtained in the treatment with 10 mM kg^{-1} CA application and AMF inoculation. For different plant tissues, application of CA significantly enhanced the extracted amount of V, where V contents of plant shoot and root in CA treatments were 31.1–70.7 and 1.21–2.52 times higher than those in control and in AMF inoculation without CA treatments, respectively. Additionally, *M. sativa* accumulated a higher amount of V in roots than in shoots in all treatments.

Table 4
Vanadium accumulative characteristics of *M. sativa* grown in vanadium contaminated soil with different citric acid concentrations (0, 5 and 10 mM kg^{-1}) treated or in combination with AMF inoculation ($n = 24$).

Citric acid application levels (mM kg^{-1})	AMF inoculation	BCF	TF	V extraction amount (mg pot^{-1})	
				Shoot	Root
0	-M	0.01 ± 0.001c	0.01 ± 0.001d	0.03 ± 0.01d	1.05 ± 0.19c
	+M	0.01 ± 0.004c	0.02 ± 0.005d	0.06 ± 0.02d	1.63 ± 0.15b
5	-M	0.52 ± 0.05b	0.36 ± 0.02c	1.48 ± 0.22c	1.70 ± 0.14b
	+M	0.50 ± 0.02b	0.55 ± 0.04a	1.87 ± 0.16bc	1.97 ± 0.05b
10	-M	0.83 ± 0.03a	0.36 ± 0.03c	2.12 ± 0.11b	2.65 ± 0.28a
	+M	0.75 ± 0.04a	0.48 ± 0.02b	2.82 ± 0.28a	3.09 ± 0.29a

Each value indicates the mean of four replicates with its standard error. Values followed by different letters in the same column indicate significant differences (LSD, α = 0.05).

3.5 Malondialdehyde (MDA) content and antioxidant activity

The response of malondialdehyde (MDA) content and antioxidant enzymes activities such as SOD, POD and CAT in the leaves of *M. sativa* are illustrated in Fig. 5. A significant increase of MDA content in plant leaves was recorded in CA (both 5 and 10 mM) applied treatments. On the basis of CA treated plants, AMF inoculation significantly decreased the MDA content by 12.8–16.2% in the leaves as compared to their respective non-AMF inoculated plants (Fig. 5a). Three (SOD, POD and CAT) key antioxidant enzymes activities were significantly enhanced in CA application alone or in combination with AMF inoculation. Regarding to the combined effects of AMF inoculation and CA application, AMF inoculation significantly increased SOD activity by 13.0-17.3% in the leaves when compared with the respective CA treated plants without AMF inoculation (Fig. 5b). POD activities in the leaves were significantly higher than the control treatment, either in combination of AMF inoculation and CA application or alone in V contaminated soil (Fig. 5c). AMF inoculation improved the activities of CAT with no significant differences at three (0, 5 and 10 mM kg^{-1}) levels of CA application when compared with their respective controls (Fig. 5d).

3.6 Soil chemical speciation of V and pH

Table 5 shows soil chemical speciation of V and pH in *M. sativa* rhizosphere under different treatments. Both single CA (5 and 10 mM) treatments significantly increased the acid-soluble V fraction by 41.7–64.8% in soil, while the reducible V fraction showed remarkable decreases of 15.1–29.2%, respectively, as compared to the control or single AMF inoculation treated soil. Moreover, combined CA application along with AMF inoculation further increased the acid-soluble V fraction compared with their respective CA application alone ($P < 0.05$). In all treatments, the amount of oxidizable and residual V fractions decreased to varying degrees, which accounted for 16.3%-18.0% and 53.7%-56.6% of total V content in all treated soil, in contrast to 17.3% and 54.3% in the original V-polluted soil, respectively. Generally, the combination of 10 mM CA application and AMF inoculation treatment tended to be more efficient for reducing oxidizable and residual V fractions. Two-way ANOVA analysis validated acid-soluble and residual V fractions to be significantly influenced by their interaction (Table 3). In terms of V removal, *M. sativa* performed surprisingly well in the 10 mM CA application and AMF inoculation combined treatment, which total V concentration in *M. sativa* rhizosphere soil decreased by 6.8% relative to that of the initial soil V concentration (1705 mg kg^{-1}). Additionally, soil pH gradually decreased with increasing CA addition levels in vanadium contaminated soil. Either 10 mM CA treatment alone or in combination with AMF inoculation significantly decreased soil pH as compared to the other treatments.

Table 5
Chemical speciation of vanadium and pH in rhizosphere of *M. sativa* grown in vanadium contaminated soil with different citric acid concentrations (0, 5 and 10 mM kg^{-1}) treated or in combination with AMF inoculation ($n = 24$).

Citric acid application levels (mM kg^{-1})	AMF inoculation	Total (mg kg^{-1})	Acid-soluble (mg kg^{-1})	Reducible (mg kg^{-1})	Oxidizable (mg kg^{-1})	Residual (mg kg^{-1})	pH (H_2O)
0	-M	1685 ± 5.9a	96 ± 4.3d	381 ± 5.2a	304 ± 3.0a	905 ± 4.2ab	6.80 ± 0.03a
	+M	1671 ± 9.0a	88 ± 4.0d	374 ± 6.3a	298 ± 3.7a	911 ± 3.6a	6.77 ± 0.08a
5	-M	1631 ± 9.6b	136 ± 6.3c	325 ± 4.7b	284 ± 5.6ab	886 ± 6.2bc	6.70 ± 0.06a
	+M	1620 ± 4.8bc	166 ± 15.8ab	289 ± 5.0c	273 ± 7.4bc	892 ± 12.7abc	6.70 ± 0.03a
10	-M	1607 ± 4.4cd	145 ± 4.7bc	295 ± 7.6c	258 ± 4.8c	909 ± 3.8a	6.42 ± 0.08b
	+M	1589 ± 5.1d	176 ± 8.3a	278 ± 7.2c	258 ± 10.9c	877 ± 2.8c	6.40 ± 0.10b

Each value indicates the mean of four replicates with its standard error. Values followed by different letters in the same column indicate significant differences (LSD, α = 0.05).

4 Discussion

4.1 CA facilitates V transport and accumulation in plant from soil

Under natural conditions, vanadium is scarily mobile in varying degree of V polluted soil, and the order of chemical special of V content is residual > reducible > oxidizable > acid-soluble fraction (Teng et al., 2011; Cao et al., 2017). In this study, the cultivated substrate collected from V mining contaminated sites predominantly dominates in residue fraction, which is in good agreement with V smelting soil in Chenxi and in Panzhihua of China (Teng et al., 2011; Xiao et al., 2015). Low bioavailability of V in soil restricts V translocation in the soil-plant system. Thus, there was lower V accumulation in shoots of *M. sativa* grown in control soil, while a relative high concentration of V was observed in plant roots (Fig. 3), suggesting *M.*

sativa accumulated a high amount of V in roots than in shoots. Previous studies have demonstrated that most plants exhibit limited V translocation from roots to shoots (Hou et al., 2013; Imtiaz et al., 2015), and V accumulates primarily in plant root could be the physiological and biochemical mechanism in alleviating inhibition of plant metabolism (Hou et al., 2013; Qian et al., 2014).

As a natural chelating agent, citric acid plays an important role in controlling the phytoavailability of heavy metals in the soil (Turgut et al., 2004; Farid et al., 2017). In the presence of CA, elevated V bioavailability was reflected by observations of increasing V uptake in plant tissues and V morphology change in the soil, and was more obvious with increased CA concentration. Both 5 mM and 10 mM CA application were efficient in solubilizing V from the soil and inducing its uptake by *M. sativa.* This may be partly because that CA chelator contains negatively charged hydroxyl or carboxyl groups and can form stable chelating compounds with positively charged V, which was more conducive to V transportation and accumulation by *M. sativa* (Wang et al., 2019). Meanwhile, increased V accumulation significantly promoted the BCF and TF values of *M. sativa* at both levels of CA addition, which is beneficial to V phytoextraction from soil to plant tissues.

In addition, the present study showed a significant effect on V morphology resulting from a large amount of acid-soluble V releasing into the soil solution and the overall decrease of reducible V in both CA treated soil, which was in line with an enhancement of the ratio of acid-soluble/reducible V fraction (Table 5). Importantly, soil pH is a key factor affecting metal fraction and bioavailability in soil (Dong et al., 2019; Wang et al., 2020). Previous studies have reported the negative correlation between soil pH and metal availability (Wang et al., 2006). Xiao et al. (2015) reported soil pH to be negatively correlated with chemical speciation of V except oxidizable fraction, and V showed more mobility in acidic soils. High levels of CA addition could decrease soil pH (Chen et al., 2003), and the same phenomenon was also found in soil amended with 10 mM CA in this present study. Regression analysis also showed a negative and significant correlation between soil pH and acid-soluble V fraction ($R^2 = 0.34$, $P < 0.01$, Fig. 6a). The decrease in soil pH could directly related to the V mobility. Thus, the lower soil pH condition accounts for higher V bioavailability, owing to affecting the dynamic equilibrium between adsorption and desorption of vanadium complexes (Dong et al., 2019; Wang et al., 2020).

4.2 AMF inoculation promotes the growth of *M. sativa* against the toxicity of CA and V

Plant biomass is considered highly sensitive growth characteristics in response to environmental stresses (Imtiaz et al., 2015). In this study, CA application significantly decreased the dry weight of shoot and root in AMF inoculated or non-AMF inoculated plants (Fig. 1b), indicating CA addition to the V contaminated soil induced toxicity in *M. sativa*'s growth. Generally, V concentration is in low concentration for most plant shoot and root (Chen et al., 2020), while in this present study plant V concentration was much higher than that of *M. sativa* reported by Yang et al. (2011) and Gan et al. (2020). The increased bioavailable V in both CA treated soil induced a significant enhancement of V concentration in shoot and root of *M. sativa* than that of in the non-CA treated plants (Fig. 3), accompanied with more MDA

production with increasing CA addition, and consequently caused oxidative damage to *M. sativa* (Fig. 5a). A similar reduction of plant biomass was also observed in the study of Turgut et al (2004), which 1.0 or 3.0 g kg^{-1} CA used in Cd, Cr and Ni polluted soil decreased total weight of *Helianthus annuus* when compared to the control soil without CA chelator. However, the effect of CA application on plant growth performance remains conflicting results, and its promotion effect was also observed in some studies (Zaheer et al., 2015; Farid et al., 2017; Wang et al., 2019). This difference may be explained by the varieties of factors such as soil structure, the presence of toxic metal-chelator complexes and chelator dosage (Begum et al., 2012; Farid et al., 2017).

In the present study, combing plants with AMF inoculation could improve the growth of *M. sativa* under CA application, owing to AMF-plant symbiotic benefits in P nutrient uptake and antioxidant defense system. AMF-plant symbiosis significantly improved P concentration both in shoot and root of *M. sativa* across three CA addition levels. The improved P acquisition may be explained by the contribution of extensive extraradical mycelium networks by AMF-plant symbiosis (Liu et al., 2015). There were significantly positive correlations between biomass and P content in shoot (R^2 = 0.35, P< 0.01, Fig. 6b) and in root (R^2 = 0.79, P< 0.01, Fig. 6c). Certainly, the improvement of P uptake associated with AMF inoculated plants was disturbed under CA chelator use, leading to the lower ratio of P/V concentration at both levels of CA addition (Fig. 4). Previous studies suggest that heavy metals show inhibition effects in nutrient absorption and metabolism in soil-plant system (Cao et al., 2009; Aihemaiti et al., 2019). Interestingly, vanadate has a similar structure and charge to phosphate, and is absorbed into plant through P uptake system which results in competition in plant uptake and assimilation between them (Chen et al., 2020). Aihemaiti et al. (2019) reported the high levels of V significantly decreased P accumulation in *Setaria viridis* seedlings. Additionally, increased P concentration in both shoot and root were also found at both levels of CA addition compared to non-CA treated plants. This was perhaps ascribed to the effective release of phosphate adhering to soil solid phase into labile fractions in the soil solution under the influence of exogenous CA addition (Wei et al., 2010; Zhu et al., 2018). Therefore, it may be reasonably concluded a joint contribution of CA chelator and AMF symbiosis leading to higher P concentration in both combination treatments, although the synergistic effect was not observed.

Previous studies have reported that high V concentration can induce an imbalance between ROS generation and scavenging in some plants, and the overproduction of ROS that interact with biomolecules inside the plant cell can cause oxidative damage (Imtiaz et al., 2018; Chen et al., 2020). Some antioxidants, including SOD, POD and CAT, are regarded as the important enzymes to eliminate the reactive oxygen species (ROS) against adverse environmental stress (Mollavali et al., 2016). In the present study, CA application stimulated the activities of three antioxidants (SOD, POD and CAT) with the increasing CA addition levels, while AMF inoculation stimulated more accumulation of antioxidants and decreased MDA production in the leaves of *M. sativa* in response to V stress (Fig. 5), which suggested that AMF symbiosis could enhance V stress resistance induced by CA addition and contribute to scavenge ROS and thus alleviate oxidative damage. Meanwhile, the lower MDA production in AMF

inoculated plants also clearly demonstrated that, the adverse effect induced by oxidative damage was to some extent counterbalanced by AMF symbiosis.

4.3 Citric mediated AMF-plant symbiosis promotes plant V phytoextraction

Soil V contamination has become a serious environmental problem and requires sustainable and environmental-friendly strategies of remediation. In this present study, we firstly demonstrated that the combined CA application and AMF inoculation treatment had more pronounced promotion in extracted amount of V in *M. sativa*, accompanied by good growth performance and enhanced antioxidant enzyme activities against V stress (Table 4), suggesting this combination could be recommended for assisting V-phytoextraction in the studied site. In the combined plants, AMF symbiosis could counterbalance the negative effects on plant biomass induced by CA chelating agent, suggesting AMF-plant symbiosis has a noteworthy potential in growth promotion against CA-induced phytotoxicity. However, CA application also provoked inhibitory effects on mycorrhizal colonization of *M. sativa* root. This was probably due to high bioavailable V toxicity which inhibited the mycorrhizal colonization rate in the presence of CA chelator. It has been shown that mycorrhizal colonization could be negatively affected by heavy metal pollution (Kanwal et al., 2015), and decreased with the increasing metal concentration (Ruotsalainen et al., 2007). Mycorrhizal colonization sensitivity to excessive metal concentration in rhizosphere may result in negative effect on the symbiotic relationship between AMF and host plant (Kanwal et al., 2015), and thus weaken the associated host plant's growth performance. Plant biomass is one of the key factors for efficient phytoextraction. In present study, mycorrhizal colonization was positively correlated with dry biomass of shoot (R^2 = 0.66, P< 0.01, Fig. 6d) and root (R^2 = 0.62, P< 0.01, Fig. 6e) in mycorrhizal plants. Thus, it should be noted to consider the concentration and application period of CA or in combination with other chelating agent capable of synergistic effect in the phytoextraction of metal (Farid et al., 2017; Wang et al., 2019).

The important factors for successful phytoextraction of metals include metal bioavailability in soil, the BCF value and plant biomass (Turgut et al., 2004; Sarwar et al., 2017; Wang et al., 2019). For the combination treatment, it showed great performance in mobilizing soil V. In both CA treated soil, its combination with AMF inoculation on V morphology was a significant increase in the acid-soluble fraction with concomitant decreases in the reducible, oxidizable and residual fraction compared with the single CA treatments. This suggests the benefit of AMF-mediated effects on the increased fraction of acid-soluble metal, which has been reported in the study of Wei et al. (2016) for antimony (Sb) and Wang et al. (2020) for cadmium (Cd), similar to our findings of V. AMF inoculation can stimulate microbial activity and secrete organic acids such as citric and oxalic acids through the mycelium and exudates, thus acidifying the rhizosphere soil (Dehghanian et al., 2018). In this study the decreased soil pH may be account for the increase of soil acid-soluble V fraction in the CA and AMF symbiosis combination soil.

However, plant shoot and root V concentration was lower in the combined treatment than that of in single CA application treatment (Fig. 3). The role of AMF symbiosis on metal uptake in contaminated soil-plant

system has been systematically studied, with some studies reporting improvements, some reporting suppression and others indicating no discernible effect on metal uptake. These contradictory results may be ascribed to some factors such as AMF species, soil properties, plant species and metal type and concentration in the soil (Miransari, 2011). Chen et al. (2004) addresses the protective effects of AMF-plant symbiosis which the lower metal concentrations in mycorrhizal plants than the uninoculated plants when grown in the contaminated soil with high concentrations of metals, and this could be regarded as a resultant growth dilution effect. The dilution effect may explain the lower plant V concentration in both combination treatments in this study. Thus, AMF symbiosis had inhibitory effects on CA chelator to enhance BCF values of V by *M. sativa* (Table 3). Although the combined plants had lower BCF, it showed notable potential of V translocation from root to shoot compared with the CA alone treatment. The enhancement of TF in combined plant might have resulted from the markedly decreased V concentration in the root, which may be a defense strategy adopted by AMF-plant symbiosis through the inhibition absorption of high concentration of metals in the soil (Kanwal et al., 2015). Moreover, higher V concentration in shoot and root of *M. sativa* showed no obvious toxic symptoms. This indicates that *M. sativa* is V-tolerant plant, indicative of strong potential to assist V phytoextraction associated with the advantage of successive cultivation without repeated planting at each new cycle.

5 Conclusions

In conclusion, CA application could improve the bioavailability of both V and P in the soil and their transportation and accumulation in soil-plant system, but at the same time, CA could induce phytotoxicity to plant growth, AMF-plant symbiotic relationship as well as the decreased P/V concentration ratio. In our pot-culture system, CA application combined with AMF inoculation could improve *M. sativa*-mediated V mobility, enhance plant biomass and P accumulation, and alleviate V toxicity to plants by stimulate antioxidant enzyme activity. These effects of the combined CA application and AMF inoculation enhance the extracted amount of V from soil. Specially, in this study, 10 mM CA application combined with AMF inoculation benefited most the V phytoextraction by *M. sativa* grown in V contaminated soil. Therefore, this study provides a new understanding of citric acid-AMF-plant symbiosis in phytoextraction of V as well as the competitive absorption between P and V. The findings also shed light on the potential application of citric acid-AMF-plant symbiosis associated with suitable plants in *in situ* phytoextraction of V or other heavy metal(loid)s.

Declarations

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Compliance with ethical standards

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Data availability

All data related to this publication are made available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

Authors' contributions

Lang Qiu wrote the original draft, and Hanzhi Lin conceptualized and designed the study. Wenlong Gao, Zhigang Wang, Benru Song, Yanxu Zhang and Tianle Kong performed the experiment and data analysis. Baoqin Li, Weimin Sun, Pin Gao and Xiaoxu Sun commented on and revised the manuscript. All authors read and approved the final manuscript.

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Figures

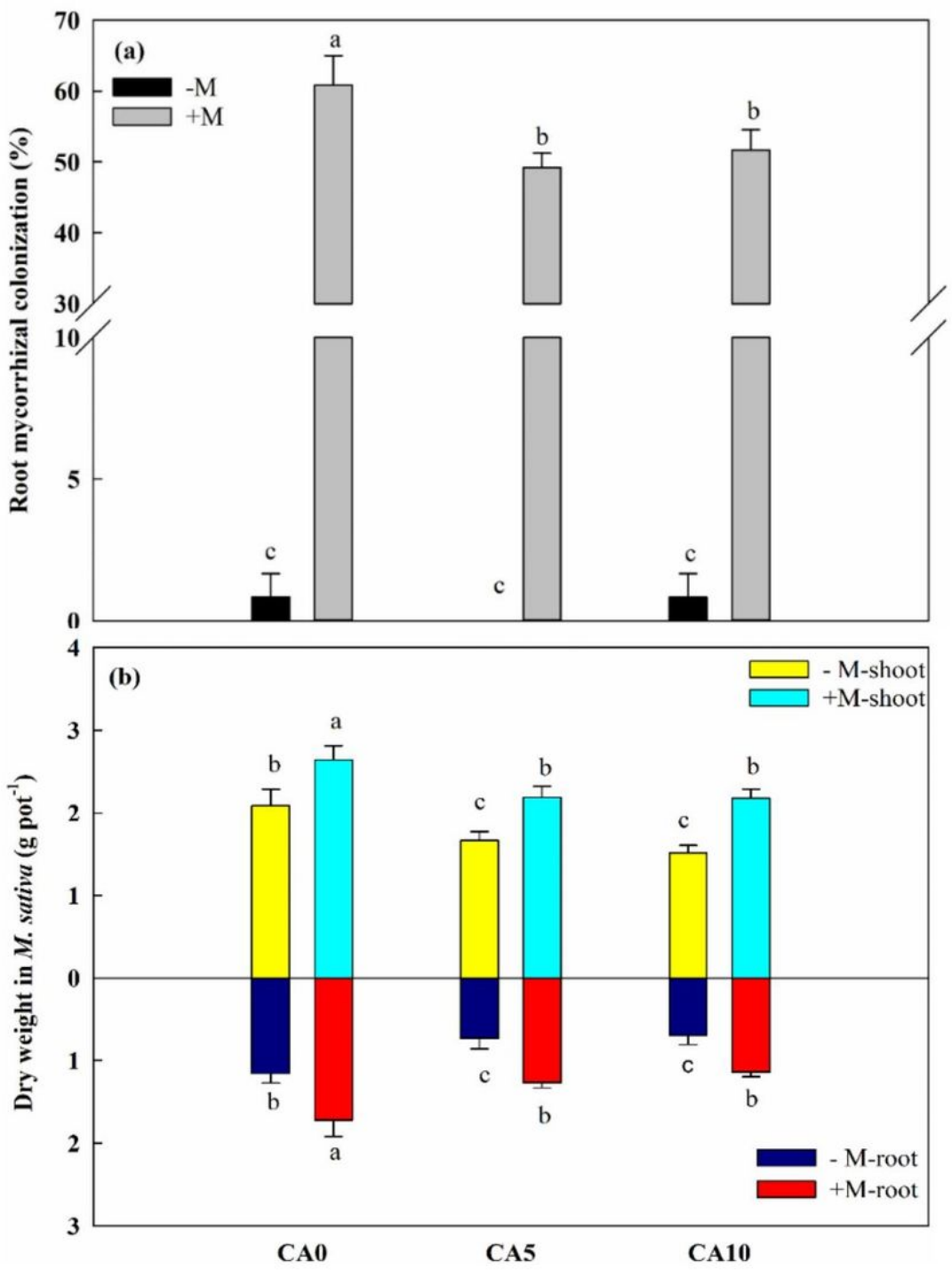

Figure 1

Effect of AMF inoculation and citric acid application on root mycorrhizal colonization (a) and dry weight (b) of M. sativa grown in vanadium contaminated soil with different CA concentrations (0, 5 and 10 mM kg-1) treated or not with AMF inoculation (+M and -M). Bars represent standard error (SE) of four replicates. Different letters between treatments indicate significant differences at $P < 0.05$.

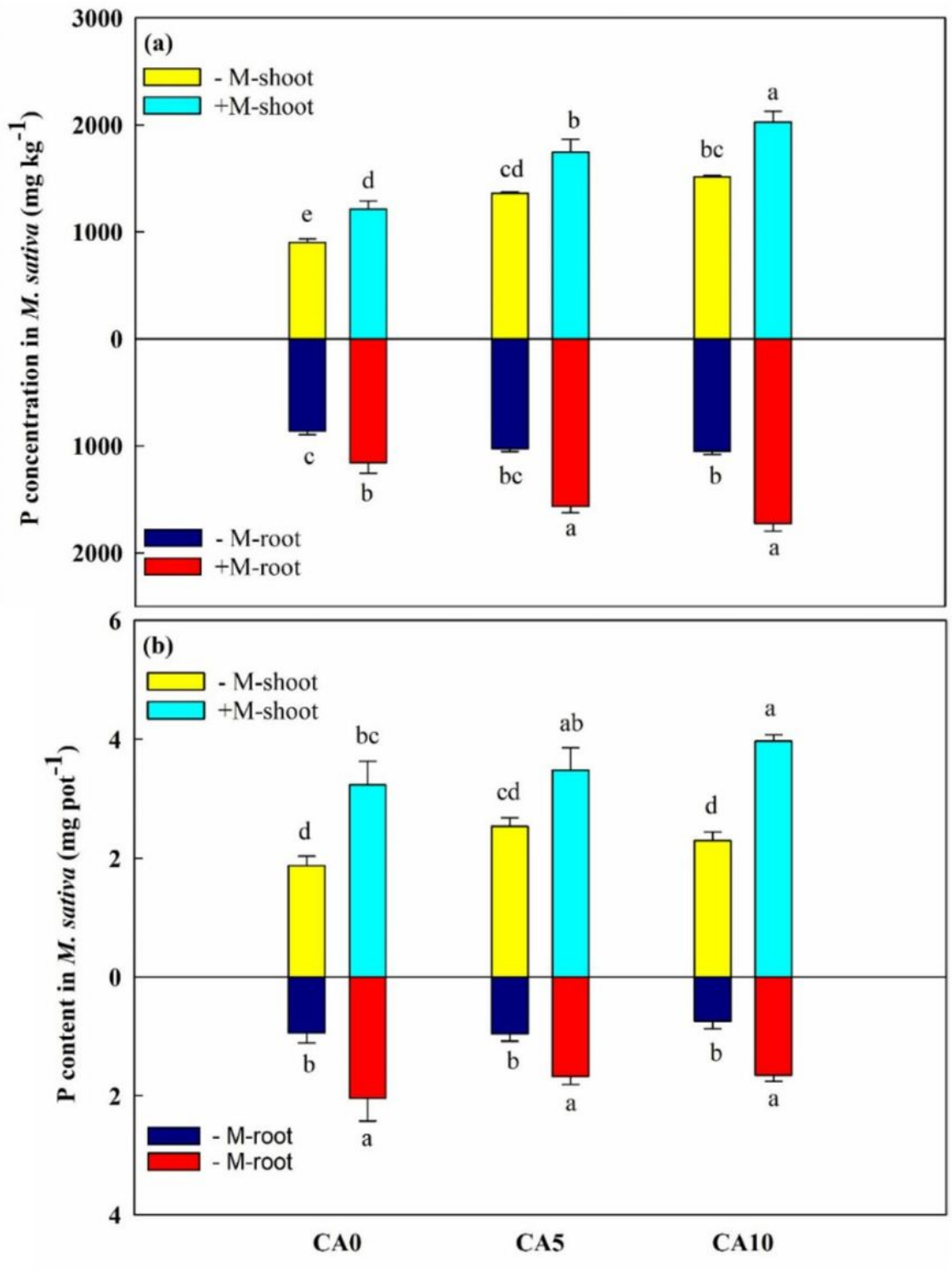

Figure 2

Effect of AMF inoculation and citric acid application on P concentration (a) and content (b) of shoot and root of M. sativa grown in vanadium contaminated soil with different CA concentrations (0, 5 and 10 mM kg-1) treated or not with AMF inoculation (+M and -M). Bars represent standard error (SE) of four replicates. Different letters between treatments indicate significant differences at $P < 0.05$.

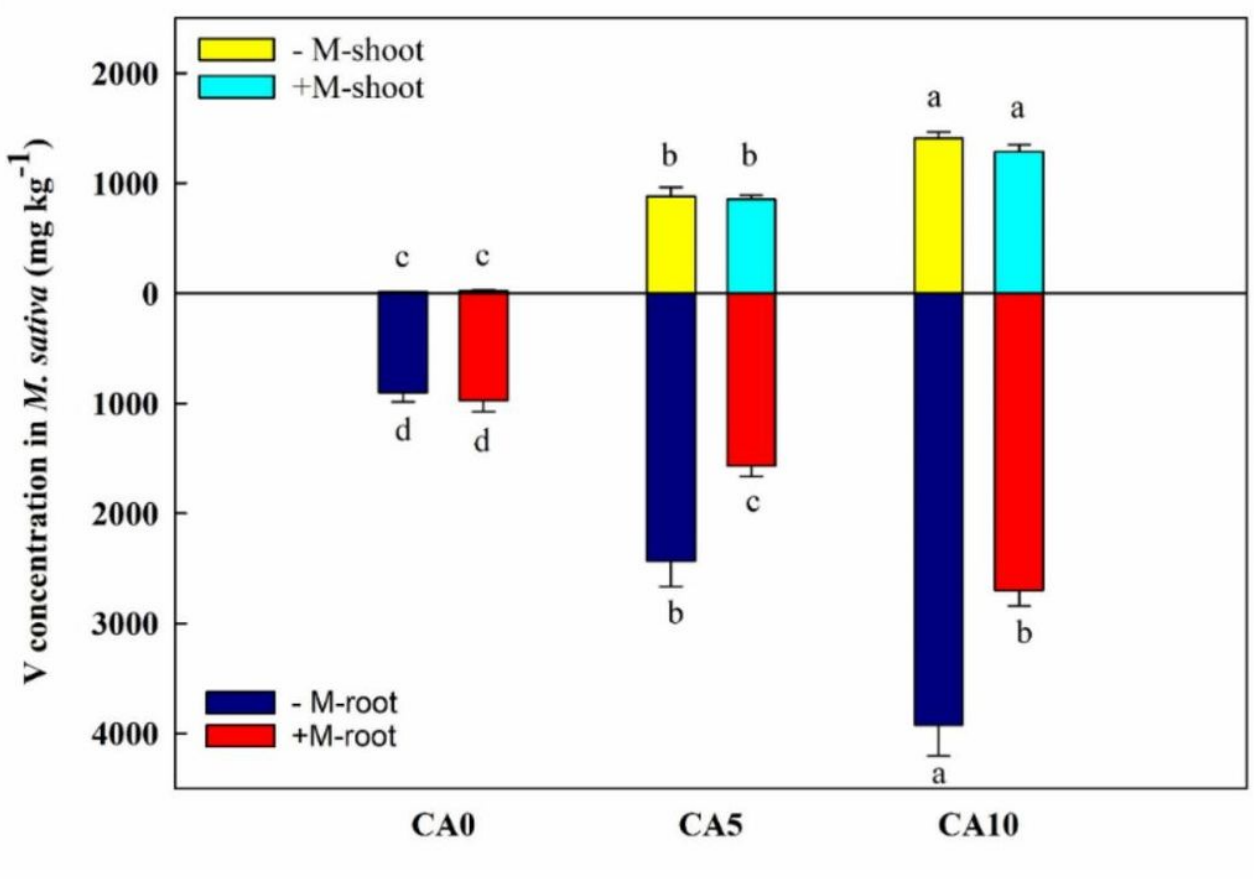

Figure 3

Effect of AMF inoculation and citric acid application on vanadium concentration in shoot and root of M. sativa grown in vanadium contaminated soil with different CA concentrations (0, 5 and 10 mM kg-1) treated or not with AMF inoculation (+M and -M). Bars represent standard error (SE) of four replicates. Different letters between treatments indicate significant differences at $P < 0.05$.

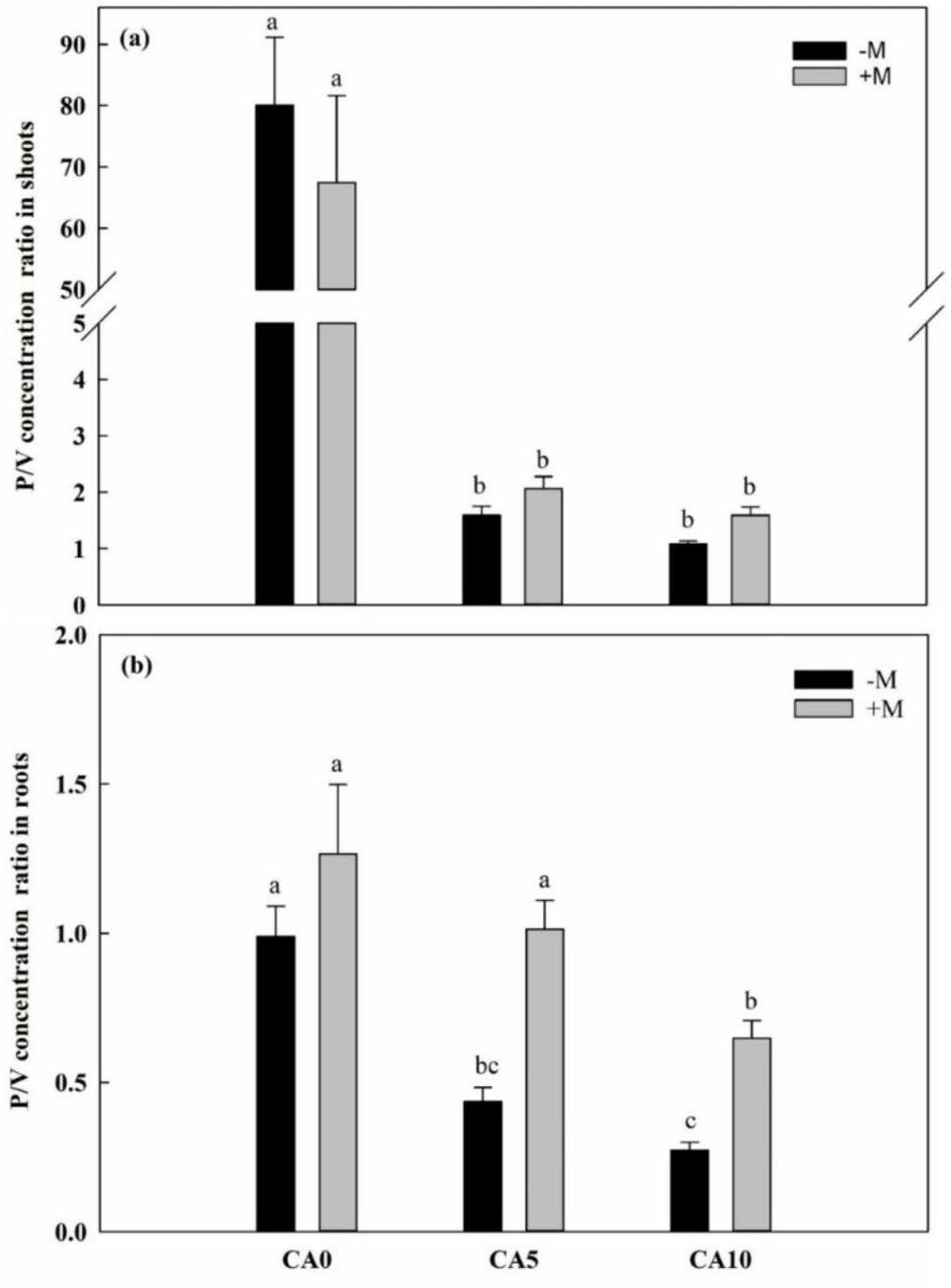

Figure 4

Effect of AMF inoculation and citric acid application on ratio of P to V concentration in shoot (a) and root (b) of M. sativa grown in vanadium contaminated soil with different CA concentrations (0, 5 and 10 mM kg-1) treated or not with AMF inoculation (+M and -M). Bars represent standard error (SE) of four replicates. Different letters between treatments indicate significant differences at $P < 0.05$.

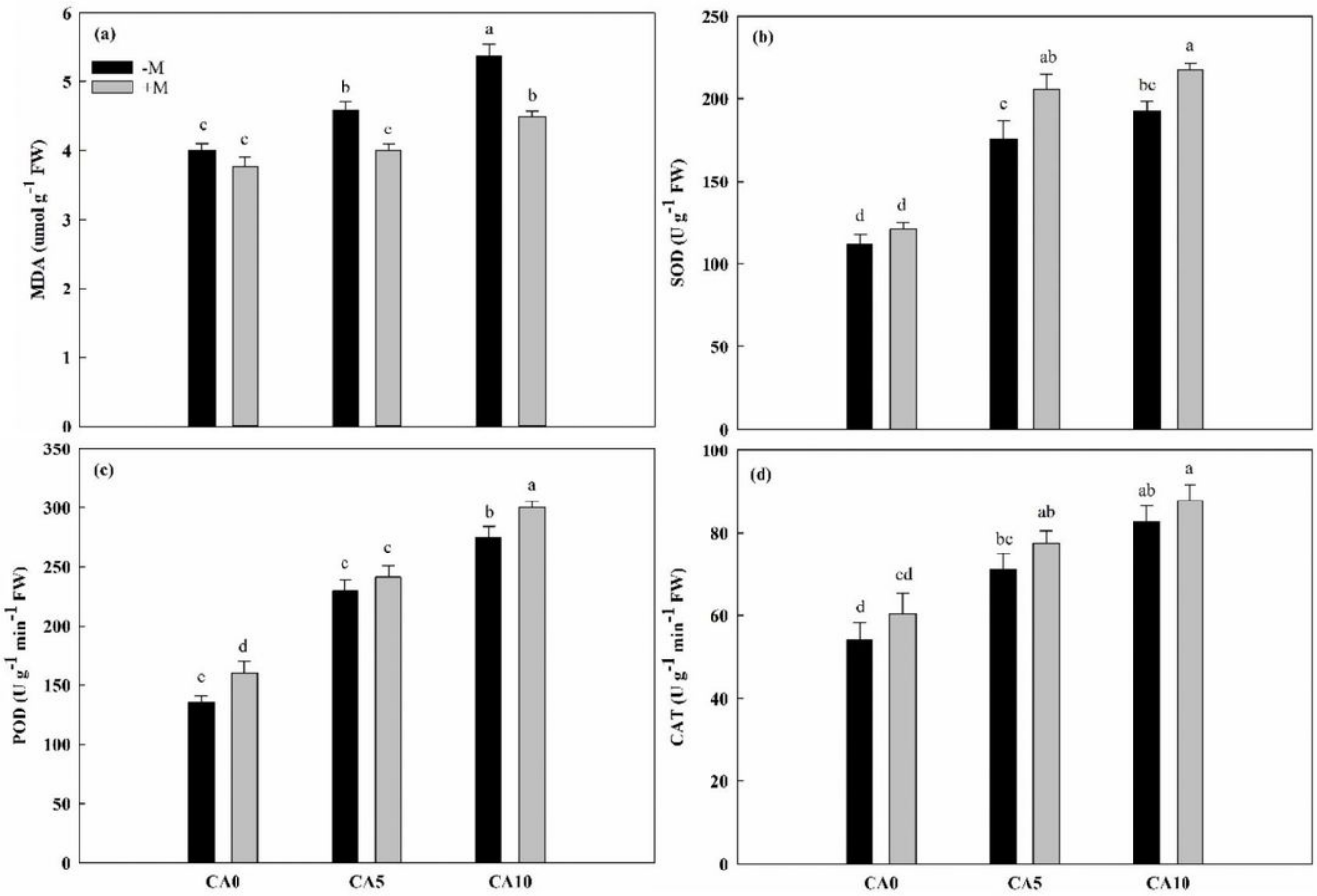

Figure 5

Effect of AMF inoculation and citric acid application on malondialdehyde (MDA) content (a) and antioxidant activities of SOD (b), POD (c) and CAT (d) of M. sativa grown in vanadium contaminated soil with different CA concentrations (0, 5 and 10 mM kg-1) treated or not with AMF inoculation (+M and -M). Bars represent standard error (SE) of four replicates. Different letters between treatments indicate significant differences at $P < 0.05$.

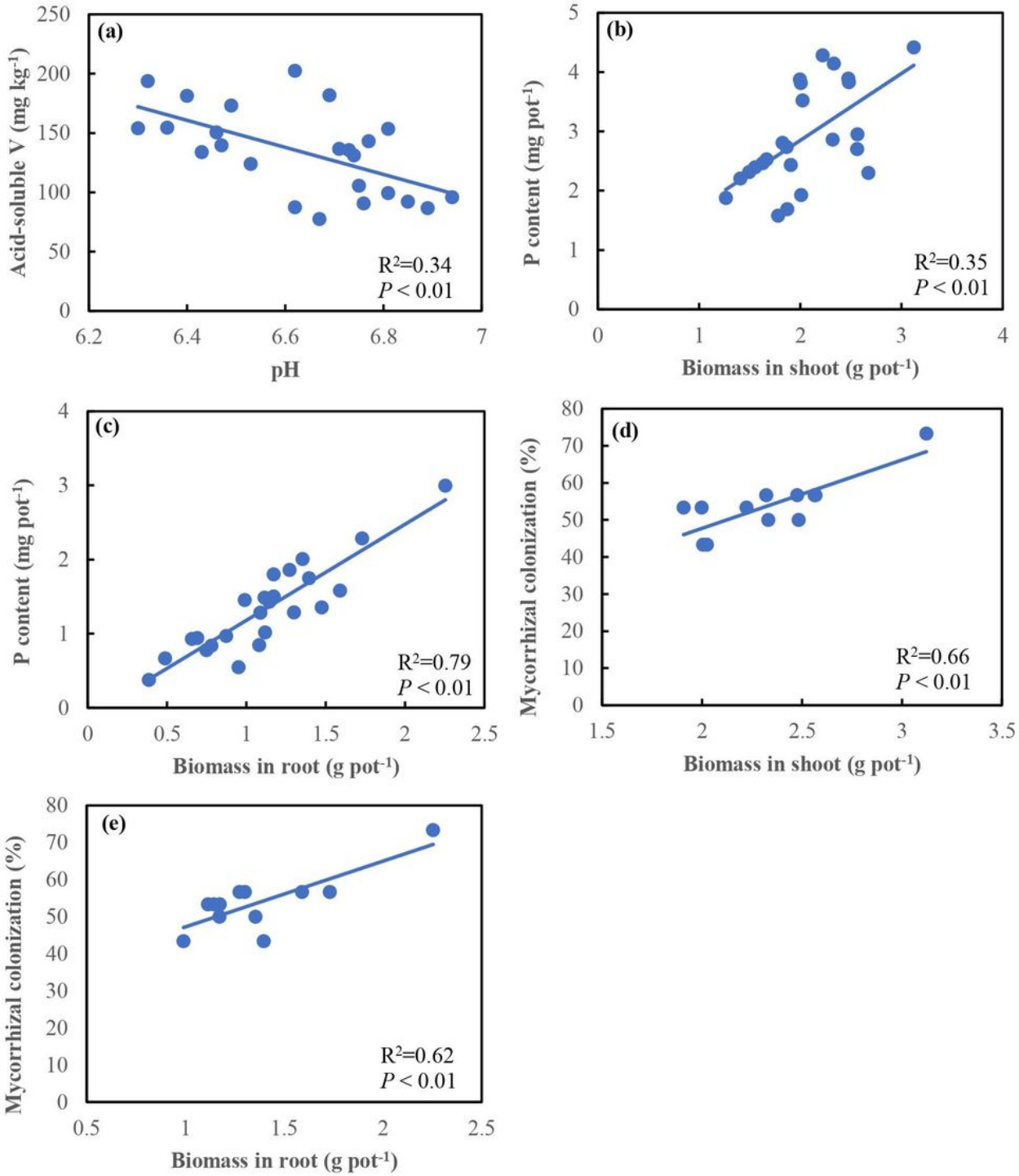

Figure 6

(a) Correlation between pH and acid-soluble V (n=24), (b) correlation between shoot biomass and P content in shoot(n=24), (c) correlation between root biomass and P content in root(n=24), (d) correlation between shoot biomass and mycorrhizal colonization in AMF inoculated plants(n=12), (e) correlation between root biomass and mycorrhizal colonization in AMF inoculated plants (n=12).

Supplementary Files

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