Arbuscular Mycorrhizal Fungi Alleviate Arsenic Toxicity in Sophora Viciifolia Hance. by Improving The Growth, Photosynthesis, Reactive Oxygen Species and Gene Expression of Phytochelatin Synthase

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Research Article

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Arbuscular mycorrhizal fungi alleviate arsenic toxicity in *Sophora viciifolia* Hance. by improving the growth, photosynthesis, reactive oxygen species and gene expression of phytochelatin synthase

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ABSTRACT: Arbuscular mycorrhizal fungi (AMF) can protect host plants against arsenic (As) toxicity. However, knowledge on the response of woody leguminous under As stress is limited so far. In this study, *Sophora viciifolia* seedlings were inoculated with/without AMF *Rhizophagus intraradices*, and *S. viciifolia* were grown in three levels (0, 50, and 100 mg As kg^{-1} soil) of As-polluted soil though the potted experiments. The objective of this study was to investigate the influences of AMF symbiosis on woody leguminous under As stress. Some physiological and biochemical parameters of *S. viciifolia*, which included the plant growth, photosynthesis, oxidative damage, antioxidant enzyme activities and gene expression of phytochelatins (PCs), were analyzed. The results showed that As toxicity in soils inhibited the AM colonization rate, plant growth, photosynthesis, increased the oxidative damage and antioxidant enzyme activities, and up-regulated the gene expression of SvPCS1 in the leaves and roots of *S. viciifolia* seedlings. However, compared with non-inoculated *S. viciifolia* at the same As level, *R. intraradices*-inoculated *S. viciifolia* had higher shoot and root dry weight, plant height, root length, photosynthetic rate (*Pn*), stomatal conductance (*gs*), transpiration rate (*E*), maximal photochemical efficiency of PSII photochemistry (*Fv/Fm*), actual quantum yield (*ΦPSII*), and photochemical quenching values (*qP*), as well as lower intercellular CO_2 concentration (*Ci*) and non-photochemical quenching values (*NPQ*). *R. intraradices* inoculation inhibited the malondialdehyde (MDA), H_2O_2, and $O_2^{\bullet-}$ concentrations, but improved the activities of antioxidative enzymes (SOD, POD, and CAT) in *S. viciifolia* leaves and roots. The gene expression of *SvPCS1* in the leaves and roots was obviously up-regulated by *R. intraradices* inoculation. These results demonstrated that *R. intraradices* inoculation enhanced the As tolerance of *S. viciifolia* seedlings, owing to the beneficial effects of AMF symbiosis on improving the plant growth, gas exchange, chlorophyll fluorescence, antioxidant enzymes, reactive oxygen species and gene expression of *SvPCS1* in *S. viciifolia* seedlings. *R. intraradices* is possible to get involved in the defence response of *S. viciifolia* seedlings against

As toxicity stress. This investigation got more profound insights into As tolerance mechanisms of woody leguminous associated with AMF symbiosis.

KEYWORDS: Arbuscular mycorrhizal fungi; *Sophora davidii*; arsenic stress; photosynthesis; reactive oxygen species; *PCS1* gene expression

INTRODUCTION

Arsenic (As) element is often defined as 'heavy metals' (HMs) due to its high density, and it is one of widespread trace components in the earth's crust [1]. The normal concentration of As in soil and water usually does not exceed 10 mg kg^{-1} [2, 3]. In the past decades, As contamination in the terrestrial ecosystem is getting worse due to excessive anthropogenic activities, such as mining of As ores, smelting of metal, burning of fossil fuel, irrigating croplands with As-contaminated groundwater, and using As-based agrochemical and phosphate fertilizers[4-7]. In consequence, the accumulation in agricultural products and water is continuing to rise beyond the threshold values for the dietary recommendation, and pose significant problems for human health through food chains[1]. As toxicity represents a threat to natural ecosystems, and As is a potent carcinogenic substance for humans and other organisms[3]. As ions, which are present in the rhizosphere soil of plants, are often absorbed into the root hair cells by the non-specific transporters of plasma membrane[8]. Potential effects of As toxicity on physiological and biochemical processes in plants are complex. It is generally accepted that it can chelate functionally critical domains of biological macromolecule, thus As suppress the cytoplasmic enzymatic activities and damage to the cell structures in plants[6,9].

When As ions are absorbed by plant roots from soil solution, excessive As stimulates the formation of free radicals (FR) and reactive oxygen species (ROS) in plant tissues at a molecular level. Increasing oxidation and radical chain reactions lead to oxidative damage of cell structures, suppression of photosynthesis, reduction in plant growth and yield, and even plant death[6,10]. Plants evolve correspondingly to the detoxification and repair system to alleviate As toxicity. The antioxidative enzymatic system, that produces antioxidant enzymes [e.g.

superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (POD, EC 1.11.1.9), and glutathione reductase (GR, EC 1.6.4.2)], can scavenge FR and ROS for preventing oxidative damage in plant cells[9]. In addition, plants induce the adaptive detoxification mechanisms of phytochelatins (PCs) to overcome heavy metal (HM) stress[1]. PCs are a family of thiol rich small peptide, and have the general structural formula of (γ-Glu-Cys)$n_{(2\ -\ 11)}$-Gly[5]. Tri-peptide glutathione (GSH) in plants synthesizes PCs by catalysis of phytochelatin synthases (EC 2.3.2.15, PCS) [11]. PCs chelate the HM ions, and then the formative stable PC-HM compounds are transported from the cytosol to the vacuoles of plant cell. Therefore, the HM toxicity can be neutralized and alleviated, and this process are regarded as a critical detoxification mechanism of HM in plant cells[5]. Some HM ions, such as Pb, Cd, Hg, Cu, Cr and As, can induce to PCs synthesis. Howerver *PCS* genes in some higher plants are isolated and described, and overexpression of Arabidopsis *PCS* genes from other plant species directly led to the higher HM resistance[1].

Higher plants, which are adapted to As-contaminated soils, are generally associated with arbuscular mycorrhizal fungi (AMF) [6]. More than 80% of terrestrial higher plants can establish symbiotic relationships with AMF[12,13]. AMF play an essential role as a "bridge" between plants and rhizosphere soil, which acquire carbohydrate compounds and lipids from host plants, and in return, transfer mineral nutrients (e.g., potassium (K), phosphorus (P), and nitrogen (N)) from rhizosphere soil to host plants by their arbuscules and hyphal coils[14,15]. AMF enhance mineral nutrient uptake of plant and soil quality, which improves the plant resistance to HM stress and alleviates HM toxicity in host plants[12]. "mycorrhizal immobilization" is the related universal mechanisms, which elucidate that AMF transform HM ions into less toxic forms, translocate

88 HM ions from host plants to the AM hyphae, and dilute HM concentration by increasing plant
89 biomass[15]. A considerable increase in the phytochelatins concentration was reporteded in the
90 mycorrhizal pigeonpea grown under cadmium (Cd) stress, compared with non-mycorrhizal
91 seedlings[16]. So AMF inoculation are considered to have a potential role on bioremediation in
92 HM-contaminated areas.

93 The widely recognized mechanism on AMF enhancing As tolerance of host plants is "growth
94 dilution effect," which signify that AMF increase plant P absorption, lead to growth promotion
95 and dilute As content in plant tissues[2,17]. *Hymenoscyphus ericae*, which is an ericoid
96 mycorrhizal fungi, maintain low As levels in *Calluna vulgaris* by inhibiting cellular arsenic to
97 arsenite and excluding arsenite from *C. vulgaris* cells[18]. AMF symbioses ameliorate As toxicity
98 in host plants due to lower arsenate/phosphate ratios in tissues of mycorrhizal plants, compared
99 with those of non-mycorrhizal plants[19]. AMF improve high-affinity P/As transfer into *Holcus*
100 *lanatus* roots[20]. Previous studies also verify that AM symbiosis regulates the expression of some
101 genes in host plants under As stress. AMF (*Glomus intraradices*) inoculation down-regulate the
102 expression of the HvPht1gene (encoding high-affinity inorganic orthophosphate (Pi)-uptake
103 systems in a direct pathway via root epidermis and root hairs) in *Hordeum vulgare*[21]. As a result,
104 the *G. intraradices*-inoculated seedlings decrease the uptake of As in barley roots, compared
105 with non-inoculated seedlings. Induced expression of GiPT (High-affinity Pi/As transporter)
106 gene in *G. intraradices* correlates with As uptake in AM external hyphae[22]. The AMF symbiosis
107 is known to increase the tolerance of host plants to various HM stresses, but necessary
108 information on the gene expression of *PCS* in AM-inoculated versus non-inoculated plants
109 under As stress is yet scarce.

Nowadays, phytoremediation is probably considered as one of the most promising techniques on disposing of As-contaminated water and soil, and the ideal candidate of plant species for this technology should be those with fast growth, high biomass yield, and outstanding ability to accumulate and inactivate As[1]. Fast-growing woody leguminous are recommended to use in phytoremediation in recent years[23]. *Sophora viciifolia* Hance., is a kind of vigorous perennial leguminous shrub, which widespread in warm-temperate to subtropical areas of East Asia. This species has developed root system, high survival rate, fast growth rate, and good adaptability to tolerate harsh environmental conditions, such as, nutrient depletion, and drought, salinity[11]. *S. viciifolia* is also a dominant pioneer plant that grows widely in some lead-zinc mining areas of northwest China, and it is known as an ideal species to use in vegetation restoration in Pb-contaminated areas[15]. Pb toxicity in *S. viciifolia* seedlings is alleviated by AMF (*Funneliformis mosseae*) symbiosis, and *F. mosseae* inoculation enhances the expression of *SvPCS1* gene in the roots under Pb stress[11]. In the present study, the growth, photosynthesis, antioxidant defense systems and genetic expression of *SvPCS1* were compared between non-AM and *Rhizophagus intraradices*-inoculated *S. viciifolia* under the condition of different As levels in soils. The objectives of the current study were to reveal the molecular and related physiological mechanisms on AMF improving As tolerate of *S. viciifolia* seedlings. The symbiosis between AMF and woody legumes maybe represents a good strategy for the restoration of vegetation and phytoremediation under As-contaminated soils.

RESULTS

AM colonization rate. AMF colonization was readily detected in the roots of *R. intraradices*-inoculated *S. viciifolia*, but not found in the roots of non-inoculated plants (Table 1). The

colonization rates of *S. viciifolia* by *R. intraradices* were 65.0%, 56%, and 43.5%, at 0, 50 and 100 mg kg^{-1} As levels. The addition of As to soils had a significantly negative influence on *R. intraradices* colonization in *S. viciifolia* roots, which was decreased with the increase in As concentration in soils.

Plant growth. The two-way ANOVA revealed significant effects of As level and *R. intraradices* inoculation on the growth of *S. viciifolia* seedlings (Table 1). Plants showed symptoms of As toxicity, such as leaves wilting and yellowing, when exposed to high As stress. The shoot and root dry weight, plant height, and root length of *S. viciifolia* seedlings were restrained with the increased As concentrations in soils ($P < 0.01$), except that the shoot dry weight in *S. viciifolia* seedlings and root length in *R. intraradices*-inoculated seedlings had no significant difference between 0 and 50 mg kg^{-1} As level.

The shoot and root dry weight, plant height, and root length in *S. viciifolia* seedlings were obviously benefitted by *R. intraradices*-inoculation ($P < 0.05$). *R. intraradices*-inoculated *S. viciifolia* seedlings had greater shoot and root dry weight, plant height, and root length than non-inoculated *S. viciifolia*, irrespective of the As treatment. But the root length in 0 mg kg^{-1} As level had no significant difference between *R. intraradices*- and non-inoculated seedlings.

The As concentration in *S. viciifolia* shoots and roots enhanced with the increasing As level in soils, irrespective of *R. intraradices* inoculation ($P < 0.05$). As concentration in *S. viciifolia* shoots and roots was obviously decreased by *R. intraradices* inoculation at the same As level in soils, except for that in 0 mg kg^{-1} As level. Significant interactive effects of As level × *R. intraradices* inoculation on As concentrations in the shoots and roots were also found in this study. Furthermore, As concentration in leaves had a very lower value, compared to roots at the

same As level in soils.

Gas exchange and chlorophyll fluorescence. As stress had a noticeable effect on the parameters of gas exchange in *S. viciifolia* plants ($P< 0.01$), which depressed the Pn, gs, and E, and improved the Ci (Fig 1). But there were no significant differences in the Pn between 0 and 50 mg kg^{-1} As levels in *R. intraradices*-inoculated seedlings. Inoculation with *R. intraradices* in the roots of *S. viciifolia* remarkably increased the Pn and gs and decreased the Ci ($P < 0.01$). But it had no obvious effect on the E, irrespective of *R. intraradices*-inoculation. The results of two-way ANOVA indicated the Pn, Ci and E exhibited highly significant differences with the interaction between As level in soils and *R. intraradices*-inoculation ($P<0.01$).

The Fv/Fm, ΦPSII, and qP in *S. viciifolia* leaves were obviously decreased, and the NPQ was significantly increased with enhancing As level in soils ($P< 0.01$) (Fig 2). Except that the ΦPSII in *R. intraradices*-inoculated seedlings has an apparent difference between 0 and 50 mg kg−1 As levels. Inoculation with *R. intraradices* significantly increased the Fv/Fm, ΦPSII, and NPQ, and decreased qP, compared with non-inoculated plants in the same As level ($P< 0.01$).

Oxidative Damage. The oxidative damage in *S. viciifolia* leaves and roots was positively correlated with As concentrations in soils ($P< 0.01$) (Fig 3). With the increasing As concentration in soils, MDA and $O_2^{\bullet-}$ concentrations in the leaves and roots were obviously enhanced. H_2O_2 concentrations in leaves had no obvious differences between 0 and 50 mg kg^{-1} As treatments. Still, the H_2O_2 concentration was the highest at 100 mg kg^{-1} As, under both *R. intraradices*- and non-inoculation conditions.

Inoculation with *R. intraradices* resulted in significantly less oxidative damage in *S. viciifolia* leaves and roots ($P< 0.01$). At 50 and 100 mg kg^{-1} As level in soils, the MDA, H_2O_2, and $O_2^{\bullet-}$

concentrations in leaves and roots were obviously decreased by *R. intraradices*-inoculation, except the H_2O_2 concentration in leaves at 50 mg kg^{-1} As level. At 0 mg kg^{-1} As levels, there was no different in the MDA, H_2O_2, and $O_2^{\bullet-}$ concentrations in leaves and roots between *R. intraradices*- and non-inoculation treatment, expect that the MDA concentrations in the roots were decreased by *R. intraradices*-inoculation. Furthermore, significant interactive effects of As level × *R. intraradices* inoculation on the H_2O_2 concentrations in leaves and roots and the $O^{2\bullet-}$concentrations in leaves were detected in this experiment ($P < 0.01$).

Antioxidant enzyme activities. Irrespective of whether or not *S. viciifolia* was inoculated with *R. intraradices*, the SOD, POD, and CAT enzyme activities in *S. viciifolia* leaves and roots were increased with the enhance of As level ($P < 0.01$), and they were highest at 100 mg kg^{-1} As (Fig 4). Inoculation with *R. intraradices* obviously increased the SOD, POD, and CAT enzyme activities in leaves and roots at 50 and 100 mg kg^{-1} As level ($P < 0.01$). However, at 0 mg kg^{-1} As level, only the enzymatic activities of SOD and POD in *S. viciifolia* leaves were remarkably enhanced by *R. intraradices* inoculation. Significant interactive effects of As level × *R. intraradices* inoculation on the SOD, POD, and CAT activities in *S. viciifolia* leaves and the POD and CAT activities in *S. viciifolia* roots were detected in this experiment ($P < 0.05$).

The gene expression of *SvPCS1*. *R. intraradices* inoculation had notable effects on the gene expression of *SvPCS1* in *S. viciifolia* seedlings ($P < 0.01$) (Fig 5). At the same As level, the gene expression of *SvPCS1* in the leaves and roots was obviously up-regulated by *R. intraradices*-inoculation. The As concentration in soils obviously affected the gene expression of *SvPCS1* in *S. viciifolia* seedlings ($P < 0.01$), the gene expression of *SvPCS1* in the leaves and roots was obviously up-regulated with the increased As level in soils. In non-mycorrhizal plants, the gene

198 expressions of *SvPCS1* in the leaves and roots in 100 mg kg^{-1} As levels was up-regulated by *R.*
199 *intraradices*-inoculation, but they were no obviously different between 0 and 50 As levels. In
200 *R. intraradices*-inoculated *S. viciifolia* seedlings, the gene expression of SvPCS1 in the leaves
201 and roots were gradually up-regulated with the increase of As concentration in soils. Significant
202 interactive effects of As level × *R. intraradices* inoculation on the gene expression of *SvPCS1*
203 were found in this study ($P < 0.01$). Furthermore, the expression level of *SvPCS1* gene in the
204 roots were significantly higher than those in the leaves at the same As level and *R. intraradices*-
205 inoculated treatment ($P < 0.01$).

206 ## DISCUSSION

207 The colonization rate of *R. intraradices* in *S. viciifolia* roots decreased with the increase of
208 the As concentrations in our study. This result was in accordance with other pot-based studies
209 using *Glycine max* L.[17], *Lolium perenne* L.[24], and *Helianthus annuus* L.[25] under As stress. In
210 contrast, some studies reported no decrease in AM colonization rate[21], and also an increase[26],
211 when the As solution was artificially added into soils. Despite this difference of AM
212 colonization rate to As stress, each AMF symbiosis conferred the benefits to host plants[6].

213 The plant performance against HM stress and the symbiosis efficiency of AMF-inoculation
214 could be visually reflected by plant biomass[27]. High levels of As in soils often jeopardized
215 normal plant growth with toxicity symptoms like biomass decrease, stagnation in plant growth,
216 wilting, and necrosis of leaf blades[28]. In our study, the growth parameters of *S. viciifolia*
217 seedlings were restrained with the increasing the As concentration in soils. The lower AM
218 colonization rate in As contaminated soils did not mean that the symbiosis was not effective,
219 and *R. intraradices*-inoculated *S. viciifolia* grew better than non-inoculated seedlings at all As

levels, which suggests that the *R. intraradices* symbiosis played a positive role in mitigated As stress in *S. viciifolia* seedlings. AMF hyphae colonized inside root cells and formed vesicle and arbuscular structures, they enhanced As binding in roots, and restrict its further translocation to shoots, thus inhibiting As toxicity[12]. Compared with the non-inoculated controls, inoculation with *Glomus mosseae* decreases As accumulation in *Trifolium repens* and *Lolium perenne* seedlings and this inoculation resulted in greater plant biomass and more As tolerance of host plants[24]. Enhanced parameters of plant growth under As stress were closed due to improved mineral nutrition through AM extraradical hyphal networks[16,29]. In the present study, As concentrations in the shoots and roots of *S. viciifolia* seedlings increased proportionately with the As level added into the soils, however, As concentrations in the roots and shoots of *R. intraradices*-inoculated *S. viciifolia* were obviously lower than those of non-inoculated seedlings, which indicated that *R. intraradices* symbiosis decreased the As accumulation in *S. viciifolia* seedlings. The greater biomass of AMF-inoculated plants diluted the toxic metalloid and toxic effect of HM in plants[27]. AMF commonly contributed to plant growth by alleviating HM toxicity, which was also closely related to improving antioxidant defence for host plants[30].

When higher plants were exposed to As stress, higher production of $O^{2\cdot-}$, OH-, perhydroxyl (HO^{2-}), singlet oxygen ($1O^{2-}$), and H_2O_2 resulted from the transform of As(V) to As(III)[6,31]. The excessive production of ROS caused the critical oxidative damage to plant cells, which ultimately resulted in growth retardation and early senescence of plants[32]. The MDA, H_2O_2, and $O_2^{\bullet-}$ levels in *S. viciifolia* leaves and roots was positively correlated with As levels in soils in this study. As uptake triggered oxidative damage resulting in increased production of H_2O_2 and lipid peroxidation[33]. To avoid oxidative damages under As stress, plants were equipped with

antioxidant enzymes system that copes with the accumulation of ROS in plants; for example, SOD catalyzes $O2^{\bullet-}$ into H_2O_2, the latter was further transformed into H_2O and O_2 by CAT[6,31]. The SOD and CAT activities of *Populus euphratica* seedlings grown in Pb-contaminated soil were increased by *Funneliformis mosseae* inoculation[27]. Similarly, *Rhizophagus irregularis* inoculation increased the antioxidase activities in *Cichorium intybus* which grown in Zn, Pb and Cd contaminated soils[34]. A higher concentration of As increased the activities of antioxidative enzymes in *Vigna mungo* L.[28].

AMF could enhance the plant's tolerance to As by increasing the antioxidant enzymes, including SOD, CAT, and POX, and mediating the ROS scavenging activities. In this study, *R. intraradices* inoculated *S. viciifolia* displayed lower H_2O_2, $O_2^{\bullet-}$ and MDA levels, and showed higher antioxidant activities of SOD, CAT, and POD than non-inoculated seedlings. It indicated that AMF triggered a reduction in oxidative damage and an increase in antioxidant enzymes induced by As stress in *S. viciifolia* seedlings. A decrease in oxidative damage and an improvement in antioxidant enzymes are the key strategies by which AMF protected host plants against As stress[6].

The decrease in plant growth under As stress was attributed to suppression in the photosynthetic capacity, the previous studies on the effect of As on photosynthesis were mainly focused on measuring the content of chlorophyll-a and -b[6]. The activity of photosystem II and chlorophyll synthesis in plants were often inhibited by As stress[4], but the effect of AMF on photosynthetic capacities under As stress remained mostly unexplored. In this study, As stress depressed the *Pn*, *gs*, and *E*, and it enhanced the *Ci* in plant leaves. A high As concentration in soils often lead to a decrease of chlorophyll content and a change in the chloroplast shape. As

a consequence, gas exchange in higher plants was inhibited [28]. Inoculation with *R. intraradices* increased the Pn, gs, and E. It decreased *Ci* in *S. viciifolia* leaves compared with those of the non-inoculated plants in this study. The Pn increment by AMF inoculation was possibly related to a higher *gs* in AMF-inoculated seedlings than that in non-inoculated plants[27]. The effects of *R. intraradices* inoculation on the photochemical capacities of *S. viciifolia* leaves under As stress were evaluated by using a chlorophyll fluorescence analysis. The Fv/Fo value, which represented the photochemical capacity of PSII in plants, indicated the quantity and size of the active photosynthetic centers in the chloroplasts[11]. In our study, the Fv/Fm was higher than 0.8 in *R. intraradices*-inoculated seedlings without As stress. Still it was less than 0.8 in all of the plants under As stress, which indicated that As stress caused chronic photoinhibition and impaired the photochemical activity in *S. viciifolia* leaves[4]. The PSII and qP decreased with the increasing As concentrations, but they were higher in *R. intraradices*-inoculated seedlings than in non-inoculated seedlings. Moreover, our study showed that As stress distinctly inhibited the electron transport rate of PSII, but mycorrhizal symbiosis obviously improved the electron transport rate. The central Mg^{2+} of chlorophyll in the chloroplast can be replaced by some HM ions, preventing light-harvesting and causing impairment of photosynthesis. AMF enhanced the chlorophyll concentration and alleviated the negative influence of HM stress on photosynthesis in host plants[11]. Our results implied that the parameters of gas exchange and chlorophyll fluorescence in *S. viciifolia* leaves were improved by inoculation with *R. intraradices* under As stress.

PCs played a pivotal role in homeostasis, immobilization and transportation for some HMs (e.g. Cu, Pb and Zn)[11]. In this study, the *SvPCS1* expression in *R. intraradices*-inoculated

286 seedlings at 0 mg kg^{-1} As level was detected, it indicated that a essential synthesis of PCs in
287 plant cells occurred under no As stress condition. Enzyme phytochelatin synthase (PCS) could
288 also constitutively expressed in plants under normal growth conditions[4]. The gene expression
289 of *SvPCS1* in *S. viciifolia* leaves and roots was obviously up-regulated with the increased As
290 level in soils. Elevated As concentrations and the present GSH in the cytoplasm of plants
291 activated the *PCS* enzyme, which starts the PCs biosynthesis[1]. Furthermore, exposure to high
292 As stress-induced the overexpression of *PCS* catalysing the formation of PCs, the PCs-As
293 complex, in turn alleviate the stress by chelating and transferring As ions from the cytoplasm
294 into vacuole[4]. But the *SvPCS1* expression was down-regulated under Pb stress, which was
295 attributed to cell damage and expression of *AmMT2* (*Avicennia marina* Forsk., a
296 metallothionein-encoding gene) after long term exposure to Pb toxicity[35]. The regulating
297 expression of *PCS* gene as per the requirement of resilient defence against different HMs were
298 intricate in other species and growth stages of plants[4].

299 The significant cell re-organization was accompanied in the process of AMF colonization in
300 the root cells of host plant[11]. Mycorrhiza-specific signal stimulation in arbuscular colonized
301 cells operated with a cell-autonomous fashion, and the cortical cells around the infected cells,
302 were also perceived this signal stimulation with cell non-autonomous style which altered the
303 gene expression levels and transcriptome patterns in the host plants by AMF symbiosis[36,37]. In
304 the present study, the gene expression of SvPCS1 was found in both *R. intraradices*- and non-
305 inoculated seedlings, which indicated that other sort of signal pathway for regulating the
306 expression of *SvPCS1* might be induced by the chelated As in the AMF- and non-colonized
307 cells. *G. mosseae* and *R. intraradices* inoculation up-regulated the *PaPCS* (*Populus alba* L.)

expression under Cu and Zn stress, and the expression level of this gene was eight times higher than the control[38]. *G. mosseae* induced the gene coding for phytochelatin synthase and caused the up-regulation of *PaPCS* in the leaves of *Populus alba L*. grown on a multimetal (Cu and Zn) contaminated soil[39]. In our study, *R. intraradices*-inoculation obviously up-regulated the gene expression of *SvPCS1* and significantly decreased As concentration in *S. viciifolia* shoots and roots at the same As level, which was attributed to the lower As toxicity in the cytoplasm of *R. intraradices*-inoculated seedlings compared with non-inoculated seedlings. It was possible that *R. intraradices* symbiosis changed the expression of *SvPCS1*genes involved in HM chelation and transport to the vacuole. The direct effect was that AMF alleviated the As toxicity to *S. viciifolia* seedlings through "dilution effect" and stored As in the cell wall and vacuole of AMF hyphae[11].

CONCLUSION

In this study, excessive As concentration in soils had multiple effects on various physiological and biochemical parameters of *S. viciifolia* seedlings. However, *R. intraradices* symbiosis enhanced plant biomass, and improved gas exchange and chlorophyll fluorescence in *S. viciifolia* seedlings, which indicated that AMF improve the plant growth under As stress. Furthermore, *R. intraradices* inoculation increased the activities of antioxidant enzymes and up-regulated the gene expression of *SvPCS1*in *S. viciifolia* seedlings, which showed that *R. intraradices* alleviated the As toxicity and decreased As accumulation in *S. viciifolia* seedlings. Thus, this investigation demonstrated the significant role of AMF in the resistance of host plants to As toxicity. AMF inoculation was recommend as a potential contributor to alleviate As stress in leguminous species. Further studies would focus on the cooperative mechanism of PCS gene

expression in both AMF and host plants under As toxicity stress.

MATERIALS AND METHODS

Experimental design. This experiment consisted of six treatments and was set up in a complete randomized block design with two factors: (1) AMF treatments, i.e., *R. intraradices* and a non-AM inoculated control; (2) three As levels in soils, i.e., 0, 50, and 100 mg As/kg dry soil. According to Risk Control Standard of Soil Pollution in Agricultural Land in China (GB 15618-2018), when the As concentration in farmland soil was higher than 100 mg/kg, it was considered high risk and was forbidden to plant agricultural products. Each of the six treatments had five replicates; thus there were a total of 30 pots (one seedling per pot).

Growth Substrate. Farmland topsoil (5–20 cm) was collected from the campus of the Henan University of Science and Technology (HAUST), Henan Province, China. Then, the soils were mixed with the sand and organic matter (soil, sand, and organic matter 3:1:1, v/v/v). 5.65 g Na_3AsO_4 •12 H_2O was dissolved in 1 L pure water, and the 0, 50 and 100 mL arsenic solutions were respectively added into per 1 kg dry soil mixture, which was stirred fully with the blender. Eventually, three As concentrations in soils (0, 50, and 100 mg As per kg dry soil) were prepared. For this potted experiment, the soil mixture was autoclaved for 2 h at 121 °C and 0.11 MPa prior to use.

Plant Material and Growth Conditions. *Sophora viciifolia* seeds were collected in November 2016 from Shimen Realgar Mine (N 29°38′32″, E111°2′17″), Hunan Province. This mine was the largest producer of realgar in China. Plump *S. viciifolia* seeds were placed in 75% ethanol for 15 min, washed with purified water, and then sowed in autoclaved wet sand at 28 °C. After growth for 20 days, healthy seedlings were transplanted into conical frustum plastic containers

352 containing 2 kg of soil mixture. *S. viciifolia* was cultivated in a solar greenhouse, the average
353 temperature ranged from 15 to 25 °C, and the temperature was controlled through ventilation
354 system and thermal insulation quilt. The relative humidity of the growth chamber was between
355 50% and 80% from April to June 2016. All treatments received a nutrient supplement of 500
356 mL Hoagland's solution (2.0 mmol/L NaH_2PO_4)[40]. During the experimental period, 50 mL
357 Hoagland solution was supplemented weekly, and soil moisture was maintained at a field
358 capacity of 50% by applying deionized water regularly.
359 **Inoculation Treatment.** AM strain *Rhizophagus intraradices* (BGC BJ09) (N.C. Schenck &
360 G.S. Sm.) C. Walker & A. Schüßler was obtained from the Institute of Plant Nutrition and
361 Resources, Beijing Academy of Agriculture and Forestry Sciences, Beijing, China. Mycorrhizal
362 inocula comprised a mixture of AM spores (spore density of 350 per 10 g dry sand soil),
363 mycorrhizal hyphae, *R. intraradices*-infected clover root segments (average 73% AM
364 colonization rate), and sandy soil. At the time of seedling transplantation, each pot in the
365 mycorrhizal treatment was inoculated with 30 g *R. intraradices* inoculum, and 30 g of sterilized
366 inoculum was added to the non-mycorrhizal pots.
367 Parameters Measured
368 **AM colonization rate.** AM colonization in plant roots was detected on 1 cm long root
369 fragments. AM colonization was estimated after washing the collected root fragments in
370 deionized water, soaking in 10% KOH at 90 °C for 15 min, decolorizing in alkaline hydrogen
371 peroxide (3 mL NH_4OH, 30 mL 10% H_2O_2, 60 mL H_2O) for 20 min, and then acidifying in 1%
372 HCl and staining with 0.05% (w/v) trypan blue in lactophenol[41]. The AM colonization rate was
373 determined by using the grid-line intersect according to the method described by Giovannetti

and Mosse[42]. The percentage AM colonization rate was calculated as follows:

The percent of AM colonization rate =

Total number of infected roots/Total number of observed roots × 100%

Plant measurement and As concentration. After three months of growth, *S. viciifolia* seedlings were harvested. Soil adhering to the root surface was removed by using deionized water. Physiological and biochemical parameters of *S. viciifolia* were analyzed. A ruler was used to measure plant height and root length. Shoots and roots were divided to determine the separate fresh weights, and then shoots and roots were weighed after oven drying at 70 °C for 48 h to obtain the dry weight.

As content in the roots and leaves of *S. viciifolia* seedlings was extracted by nitric acid digestion at 270 °C, and determined with a graphite furnace atomic absorption spectrophotometer (Perkin-ElmerAnalyst400, Norwalk, CT, USA) following USEPA Method 7060A [2].

Gas exchange and chlorophyll fluorescence. The net photosynthetic rate (Pn), stomatal conductance (gs), intercellular CO_2 concentration (Ci), and transpiration rate (E) were measured on the fifth expanded leaf of each plant using a portable photosynthesis system LI-6400 (LI-COR, Lincoln, NE, USA). The experiment was conducted at 2,500 μmol m^{-2} s^{-1} active radiation, 350 cm^3 m^{-3} CO_2 concentration, 25.0 °C leaf temperature and 0.5 dm^3 min^{-1} atmospheric flow rate between 9:30 and 11:00 a.m. during the data acquisition period[7].

The chlorophyll fluorescence parameters were determined using a modulated PAM-2000 portable fluorometer (Imaging-PAM, Walz, Germany) on the fifth expanded leaves of *S. viciifolia*. The leaves were adapted in darkness for 1 h, and then the measurements were

conducted between 9:30 and 11:00 a.m. at room temperature. The leaves were saturated with pulse flashes of white light (2,000 μmol m^{-2} s^{-1} for 3 s), the Fo (minimum fluorescence) and Fm (maximal fluorescence) were measured for dark-adapted leaves; the Fs (steady-state) and Fm′ (maximal) fluorescence were obtained for light-adapted leaves. The Fo′ (minimal fluorescence level in the light-adapted state) was acquired by illuminating the leaves with a 3 s flash of far-infrared light (5 μmol m^{-2} s^{-1}). The maximum quantum yield of the PSII photochemistry ((Fm−Fo)/Fm) and the actual quantum yield of PSII electron transport ((Fm′-Fs)/Fm′) were calculated according to method of Genty et al.[43]. The quenching due to nonphotochemical dissipation (NPQ = (Fm - Fm′)/Fm′) and the coefficient of photochemical quenching (qP = (Fm′ - Fs)/(Fm′ - Fo′)) were calculated following previously described methods [44].

Measurement of oxidative damage. Fresh leaves or roots (1 g) were homogenized in 10 mL 10 mM sodium phosphate buffer (pH 7.4) on an ice bath, and the homogenate was centrifuged at 4,000 g for 10 min. The malondialdehyde (MDA) concentration was analyzed following the method described by Janero[45]. The rate of H_2O_2 and $O_2^{\bullet-}$ production were determined using the method previously published by Wang and Luo [46]. The absorbance of H_2O_2 in the assay mixture was spectrophotometrically determined at 390 nm. For analyzing the $O_2^{\bullet-}$ concentration, 1 mL 17 mM sulfanilic acid and 1 mL 7 mM α-naphthylamine were added in 1 mL of the mixture for 20 min at 25 °C, and then 3 mL anhydrous was used to leach chlorophyll. Concentrations of $O2^{\bullet-}$ in the assay mixture were spectrophotometrically measured at 530 nm[47].

Determination of antioxidant enzymes. To extract the antioxidant enzymes, the following steps were conducted under ice-cold conditions: 1 g of fresh leaves or roots was homogenized

418 in 5 mL 0.1 M cold Tris-HCl buffer (pH 7.6); the supernatant fraction was used after
419 centrifugation at 10,000 g for 20 min. The SOD activity (EC 1.15.1.1) was assayed
420 spectrophotometrically at 560 nm following the method described by Giannopolitis and Ries [48].
421 The amount of enzymes causing a 50% decrease of SOD-inhibitable photochemical reduction
422 of nitroblue tetrazolium (NBT) was defined as 1 U SOD activity. CAT (EC 1.11.1.6) activity
423 was measured spectrophotometrically at 240 nm according to the method of Aebi[49]. One unit
424 of CAT enzyme activity was expressed as the extinction coefficient of 1 μmol H_2O_2 oxidized
425 mg^{-1} protein min^{-1}. POD (EC 1.11.1.7) activity was assessed following the guaiacol oxidation
426 method[50]. POD was quantified spectrophotometrically at 470 nm, where 1 U POD enzyme
427 activity was the number of grams of tetraguaiacol formed per minute[7].
428 **RNA Extraction and cDNA Synthesis.** Total RNA was extracted from the fresh leaves and
429 roots by using Plant Total RNA Isolation Kit (Sangon Biotech, Shanghai, China) according to
430 the manufacturer's instructions. In order to remove residual genomic DNA, the TURBO DNA-
431 free kit (Applied Biosystems⁄Ambion) was used, and the RNA quantity was detected by using
432 using a NanoDrop 2000 (Thermo Scientific, Pittsburgh, PA, USA). Reverse transcription to
433 complementary DNA (cDNA) was operated by using a PrimeScriptTM RT reagent kit with
434 gDNA eraser (TaKaRa Bio, Dalian, China).
435 **Cloning of Partial Coding Sequences (CDSs) of SvPCS1 and SvActin.** *Sophora viciifolia*
436 cDNA was used as the template for amplifying the conserved sequences of SvPCS1 and
437 SvActin according to the method of Li et al. (2010). Two pairs of degenerate primers were
438 PCS1S (5′-GAAAGGGCCTTGGAGRTGG-3′)/PCS1A (5′-GATATDAGCATR
439 AACCCYCT-3′) and ACTS (5′-CTCCCAGGGCTGTGTTTCCT-3′)/ACTA (5′-CTCCATG

TCATCCCAGTTGCT-3′). The PCR amplification was conducted in a reaction system of 25 μl mixture containing 12.5 μl 10 ml of Premix Taq R Version 2.0, 1 μl *S. viciifolia* cDNA, 1 each primer, and 9.5 μl RNase-Free ddH_2O. The PCR reactions were conducted with a C1000 Thermal cycler (Bio-Rad, Hercules, CA, USA) with the following procedure: a 5 min denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 or 55 °C for 1 min (54 °C for SvPCS1 conserved fragment and 55 °C for SvActin conserved fragment), extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PCR products were inserted into a pGEM-T vector (Tiangen Biotech CO., LTD, Beijing, China) and transformed into Escherichia coli (strain DH5α) (Tiangen Biotech CO., LTD, Beijing, China). Then, Luria–Bertani (LB) medium amended with ampicillin was used to select the transformants. Then, in order to confirm the presence of inserts, 1 μl cultured bacteria solution was used as template DNA for PCR with primers PCSS/PCSA and ACTS/ACTA. The solutions which tested positive were used for sequencing (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd, China).

Analysis of gene expression. 2μg RNA was used to synthesize the first-strand cDNA. Reverse transcription to complementary DNA (cDNA) was conducted by using a PrimeScriptTM RT reagent kit with gDNA eraser (TaKaRa Bio, Dalian, China). For the qRT-PCR assay, 1 μg of total RNA was used for reverse-transcription, and 1 μL of the product was used in PCR amplification. The reaction system used 20 μL for the qRT-PCR assay included 10 μL of SYBR® Premix Ex Taq™ (TaKaRa), and RT-PCR was performed by using CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc., USA). Primers for qRT-PCR in this experiment are QSvPCS1S (5′-TTGTTGCCAAGGAGCAGATA-3′)/QSvPCS1A (5′-

CCTGTTTCAATACCTCTTCCTT -3′) and QSvACTS (5-GATGCTGAGGATATTCAACCC-3′)/QSvACTA (5′-TTTGACCC ATCCCAACCATAA-3′) 11. The RT-PCR amplification program of *SvPCS1* and SvActin gene was initiated at 95 °C for 3 min to activate the polymerase, followed by 40 cycles at 95 °C for 5 s and 57 °C for 30 s for getting the target gene. Three biological replicates were used for all genetic analyses, relative quantification values of *SvPCS1* gene were calculated by using the $2^{-\triangle\triangle Ct}$ method[51]. All samples were technically replicated three times. Negative controls without cDNA were runwithin each analysis.

Statistical analysis. All experimental results were subjected to a two-way analysis of variance (ANOVA) to compare As treatments and AM inoculation as the main factors. Significant differences among these treatments were evaluated by Tukey's multiple range test. Statistical analyses were conducted using SAS (SAS Institute, Cary, NC, USA). Figures were drawn with SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, United States) and the package"pheatmap" in R.

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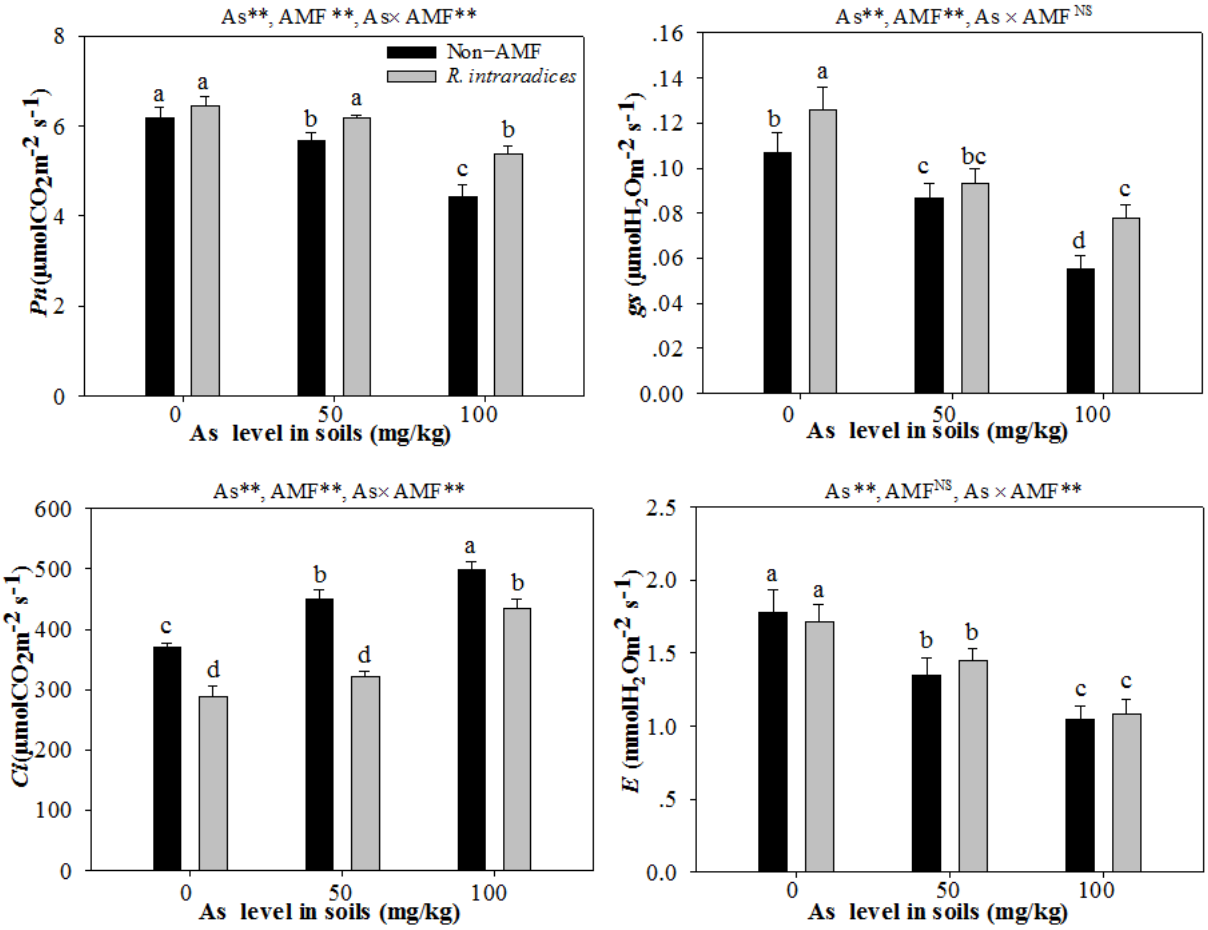
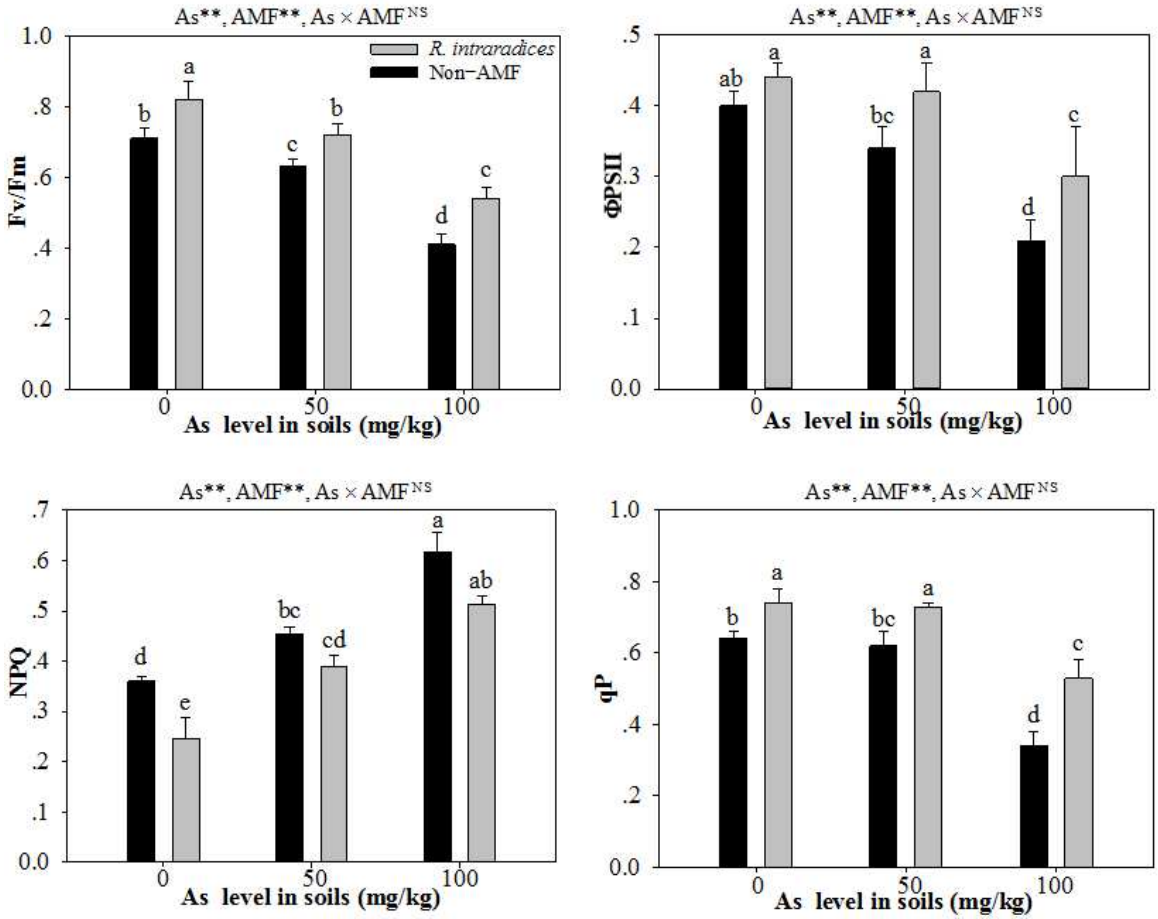
Fig 1 Effects of *Rhizophagus intraradices* on net photosynthetic rate (*Pn*), stomatal conductance (g_s), intercellular CO_2 concentration (*Ci*), and transpiration rate (*E*) in *Sophora davidii* leaves under different level of As stress



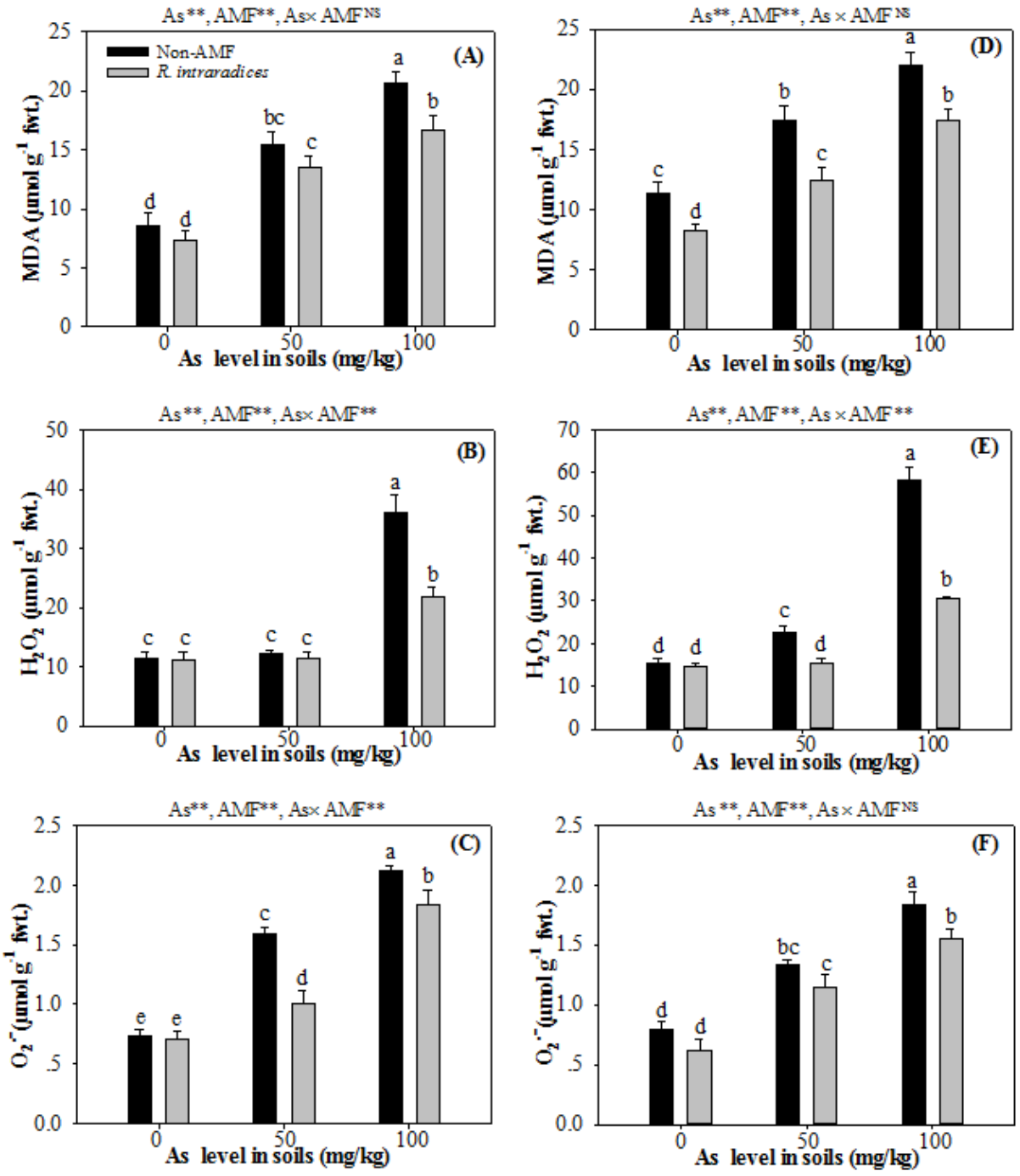
The same letter in each column indicates no significant difference among treatments at $P < 0.05$ using Tukey's test; Values are means ± SD, n = 5; *$P < 0.05$, **$P < 0.01$, NS not significant

Fig 2 Effects of *Rhizophagus intraradices* on maximum quantum yield in the dark-adapted state (*Fv/Fm*), actual quantum yield in the light-adapted steady state (*ΦPSII*), nonphotochemical quenching values (*NPQ*), and photochemical quenching values (*qP*) in *Sophora davidii* leaves under different level of As stress



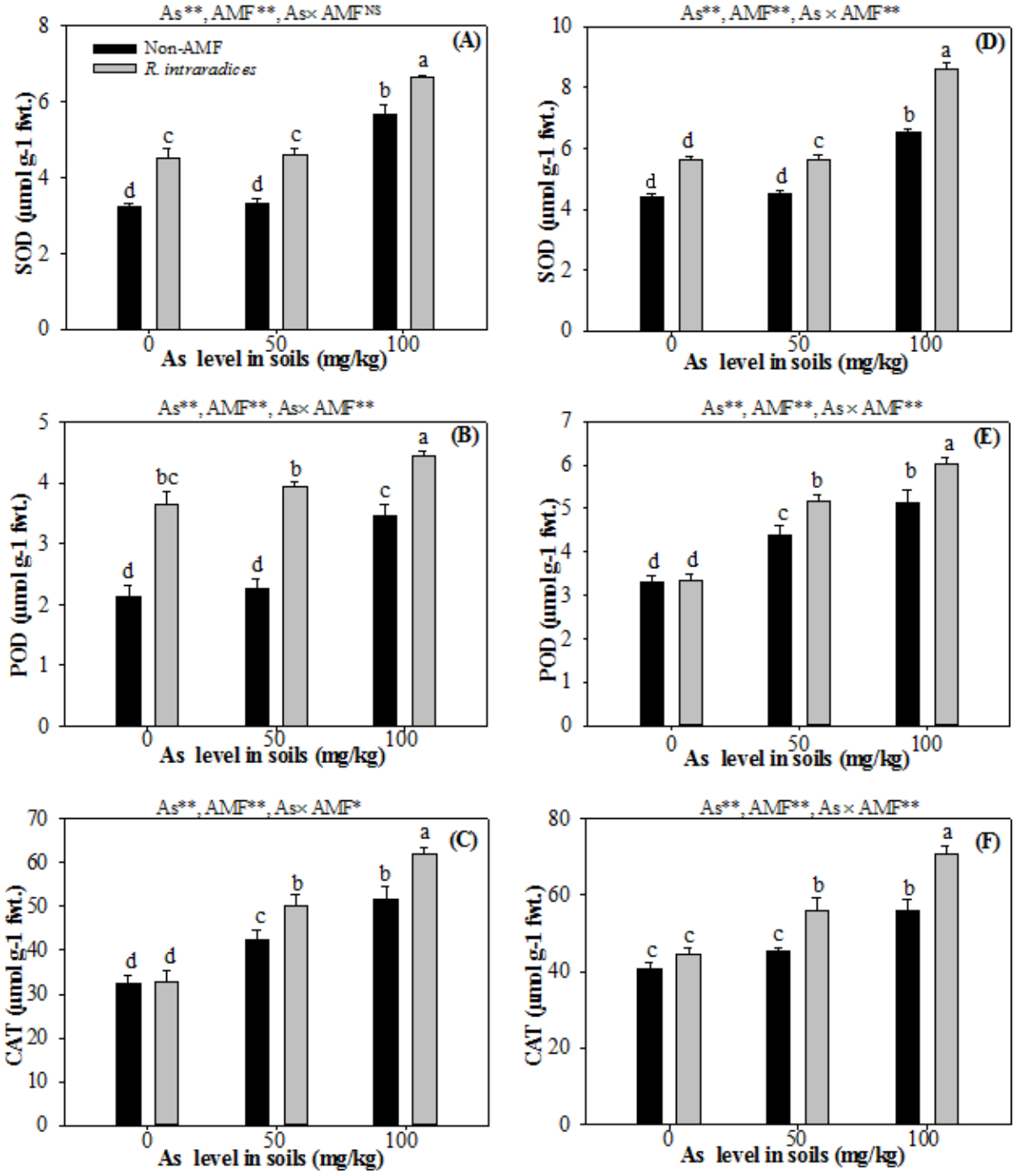
The same letter in each column indicates no significant difference among treatments at $P < 0.05$ using Tukey's test; Values are means ± SD, n = 5; *$P < 0.05$, **$P < 0.01$, NS not significant

Fig 3 Effects of *Rhizophagus intraradices* on reactive oxygen species in *Sophora davidii* leaves (A, B, C) and roots (D, E, F) under different level of As stress



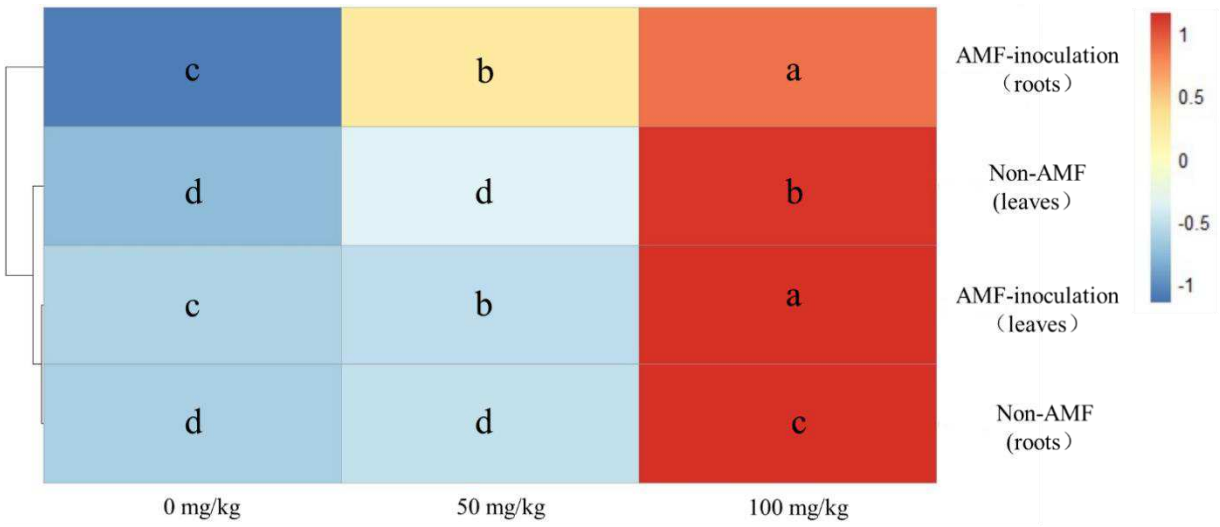
The same letter in each column indicates no significant difference among treatments at $P < 0.05$ using Tukey's test; Values are means ± SD, n = 5; *$P < 0.05$, **$P < 0.01$, NS not significant

Fig 4 Effects of *Rhizophagus intraradices* on antioxidant enzyme in *Sophora davidii* leaves (A, B, C) and roots (D, E, F) under different level of As stress



The same letter in each column indicates no significant difference among treatments at $P < 0.05$ using Tukey's test; Values are means ± SD, n = 5; *$P < 0.05$, **$P < 0.01$, NS not significant

Fig 5 Effects of *Rhizophagus intraradices* on expression of *SvPCS1* in roots and leaves of *Sophora davidii*. under different level of As stress



Different letters within each gene indicate significant differences ($P < 0.05$).

Table 1 Effects of *R. intraradices* on AM colonization, shoot dry weight, root dry weight, plant height, and root length of *S. viciifolia* seedlings under different level of As stress

Inoculation	As (V) treatments mg kg^{-1}	AM colonization (%)	Shoot dry weight (g $plant^{-1}$)	Root dry weight (g $plant^{-1}$)	Plant height (cm)	Root length (cm)	As concentration in shoots (mg/kg)	As concentration in roots (mg/kg)
	0	0	1.39 ± 0.08ab	1.44 ± 0.11ab	41.40 ±0.94ab	31.58 ± 1.16a	2.55 ± 0.22e	0.24 ± 0.11e
Non−AMF	50	0	1.38 ± 0.07ab	1.32 ± 0.13c	39.92 ±1.20ab	28.03 ± 1.37b	7.43 ± 0.48c	84.48 ± 6.84c
	100	0	0.96 ± 0.07c	1.05 ± 0.06d	35.04 ±1.50c	23.64 ± 1.31c	13.95 ± 0.32a	176.72 ± 6.51a
	0	65.0 ± 5.50a	1.53 ± 0.10a	1.54 ± 0.12a	43.85 ±1.37a	33.89 ± 1.56a	1.17± 0.29e	0.28 ± 0.05e
R. intraradices	50	56.0 ± 4.00b	1.39 ± 0.06a	1.44 ± 0.11b	42.75 ±1.45a	32.09 ± 0.93a	5.38 ± 0.38d	66.44 ± 4.23d
	100	43.5 ± 5.00c	1.21 ± 0.03b	1.22 ± 0.10c	38.67±1.29bc	27.55 ± 1.08b	10.50 ± 0.97b	138.39 ± 5.78b
Significance								
AMF		**	NS	*	*	*	NS	NS
AS			*	**	**	*	*	*
AMF × AS			NS	NS	NS	NS	**	**

The same letter in each column indicates no significant difference among treatments at $P < 0.05$ using Duncan's test; Values are means ± SD, n = 5; *$P < 0.05$, **$P < 0.01$, NS not significant

Figures

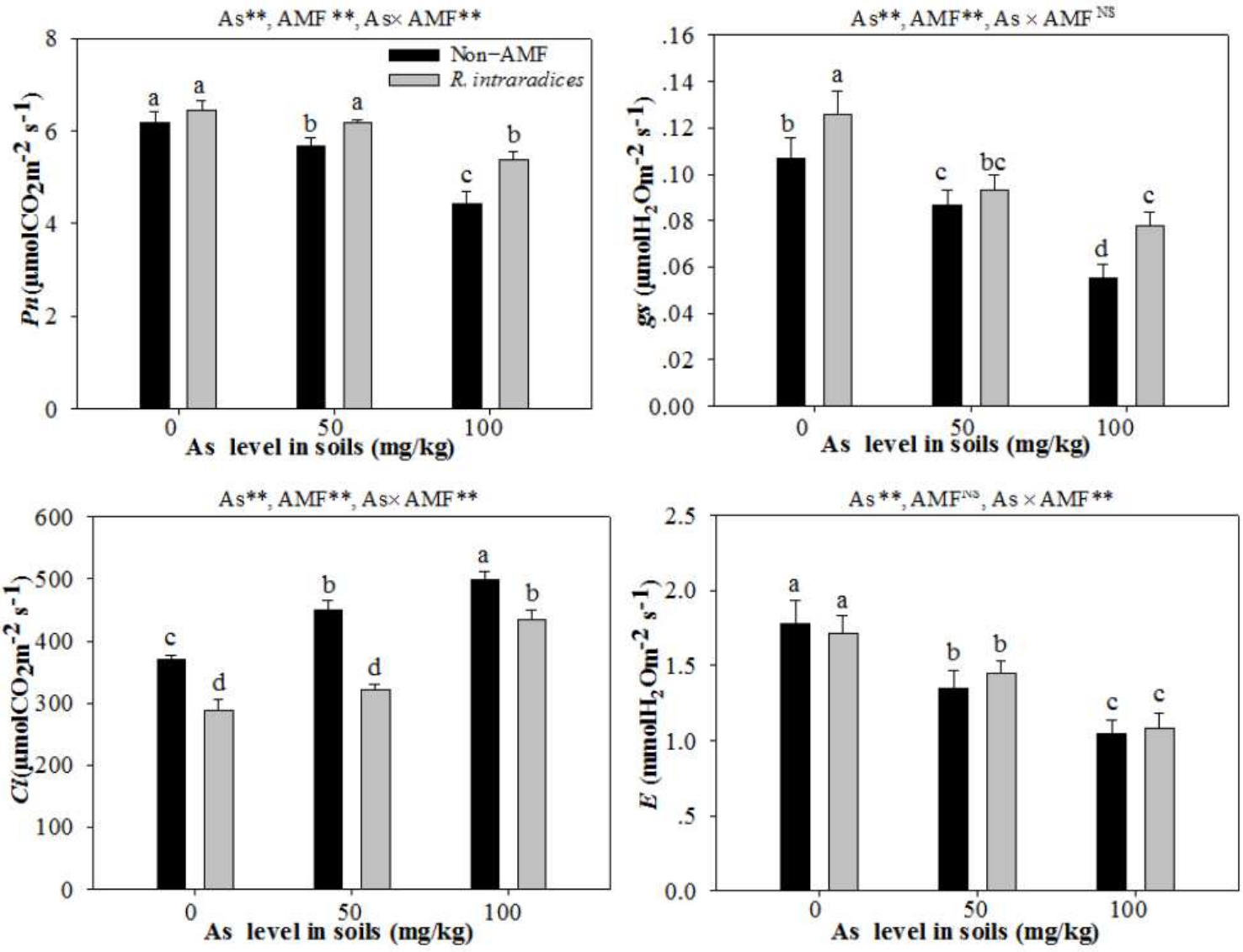


Figure 1

Effects of Rhizophagus intraradices on net photosynthetic rate (Pn), stomatal conductance (gs), intercellular CO2 concentration (Ci), and transpiration rate (E) in Sophora davidii leaves under different level of As stress The same letter in each column indicates no significant difference among treatments at $P < 0.05$ using Tukey's test; Values are means ± SD, n = 5; *P < 0.05, **P < 0.01, NS not significant

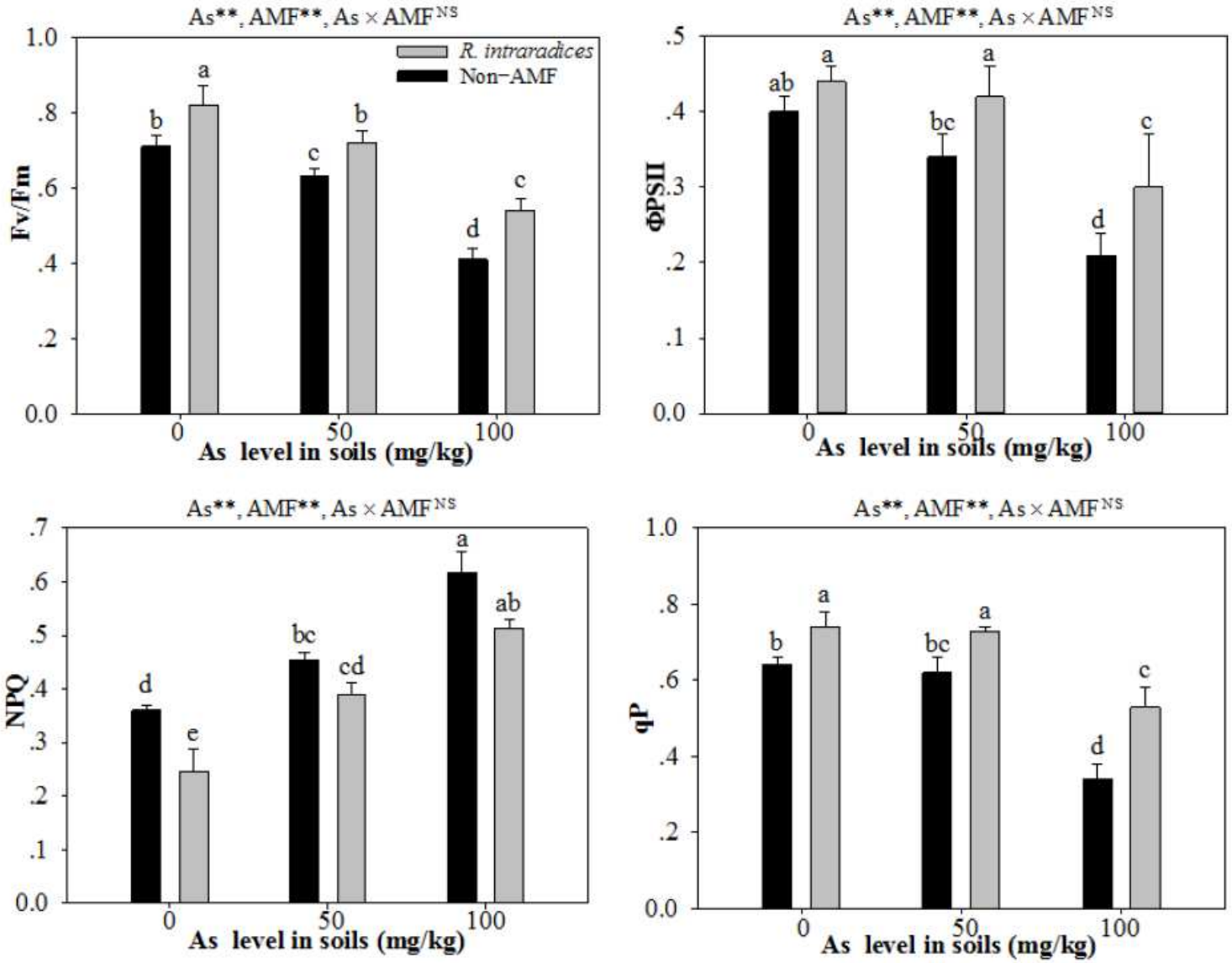


Figure 2

Effects of Rhizophagus intraradices on maximum quantum yield in the dark-adapted state (Fv/Fm), actual quantum yield in the light-adapted steady state (ΦPSII), nonphotochemical quenching values (NPQ), and photochemical quenching values (qP) in Sophora davidii leaves under different level of As stress The same letter in each column indicates no significant difference among treatments at $P < 0.05$ using Tukey's test; Values are means ± SD, n = 5; *P < 0.05, **P < 0.01, NS not significant

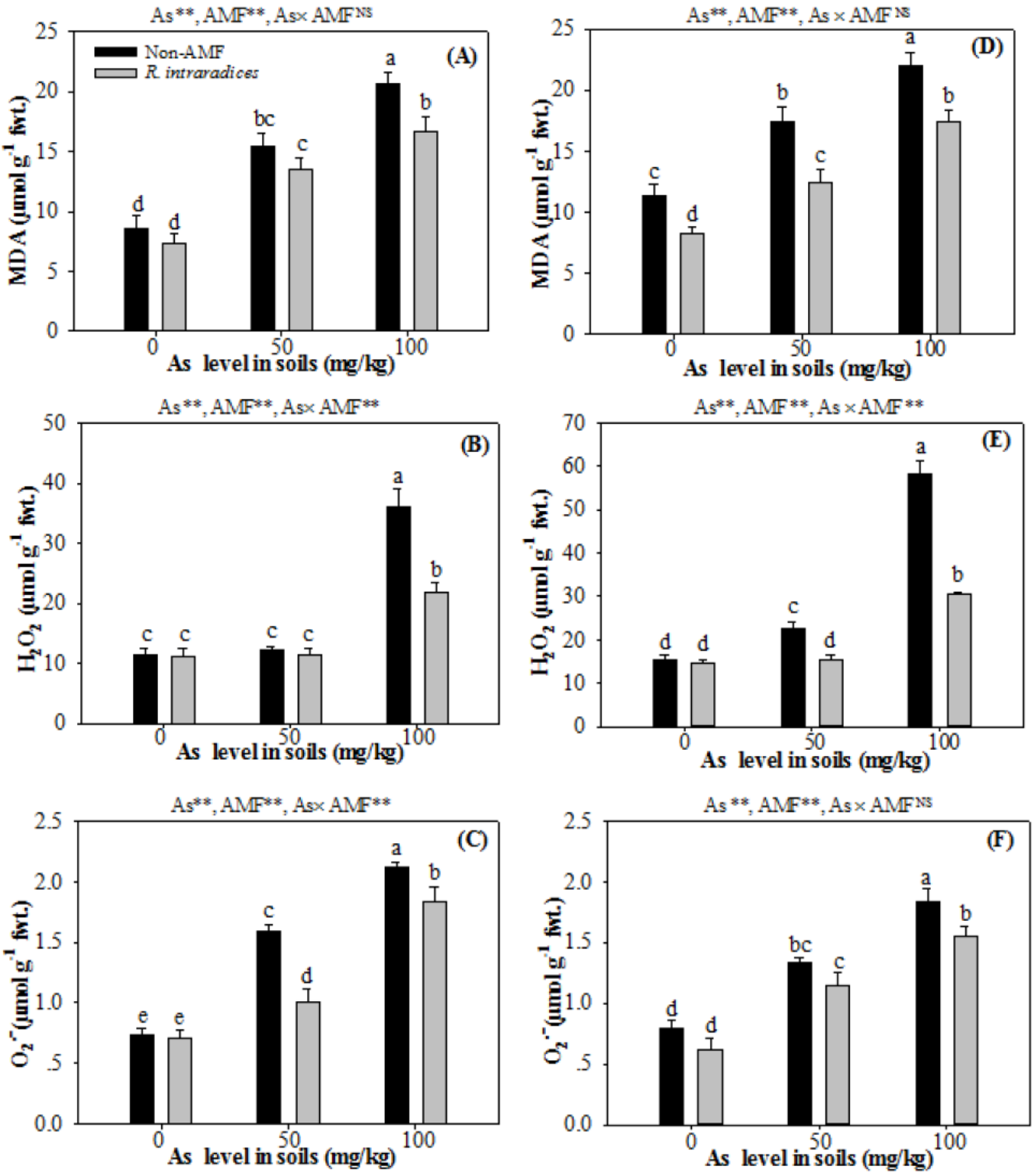


Figure 3

Effects of Rhizophagus intraradices on reactive oxygen species in Sophora davidii leaves (A, B, C) and roots (D, E, F) under different level of As stress The same letter in each column indicates no significant difference among treatments at $P < 0.05$ using Tukey's test; Values are means ± SD, n = 5; *P < 0.05, **P < 0.01, NS not significant

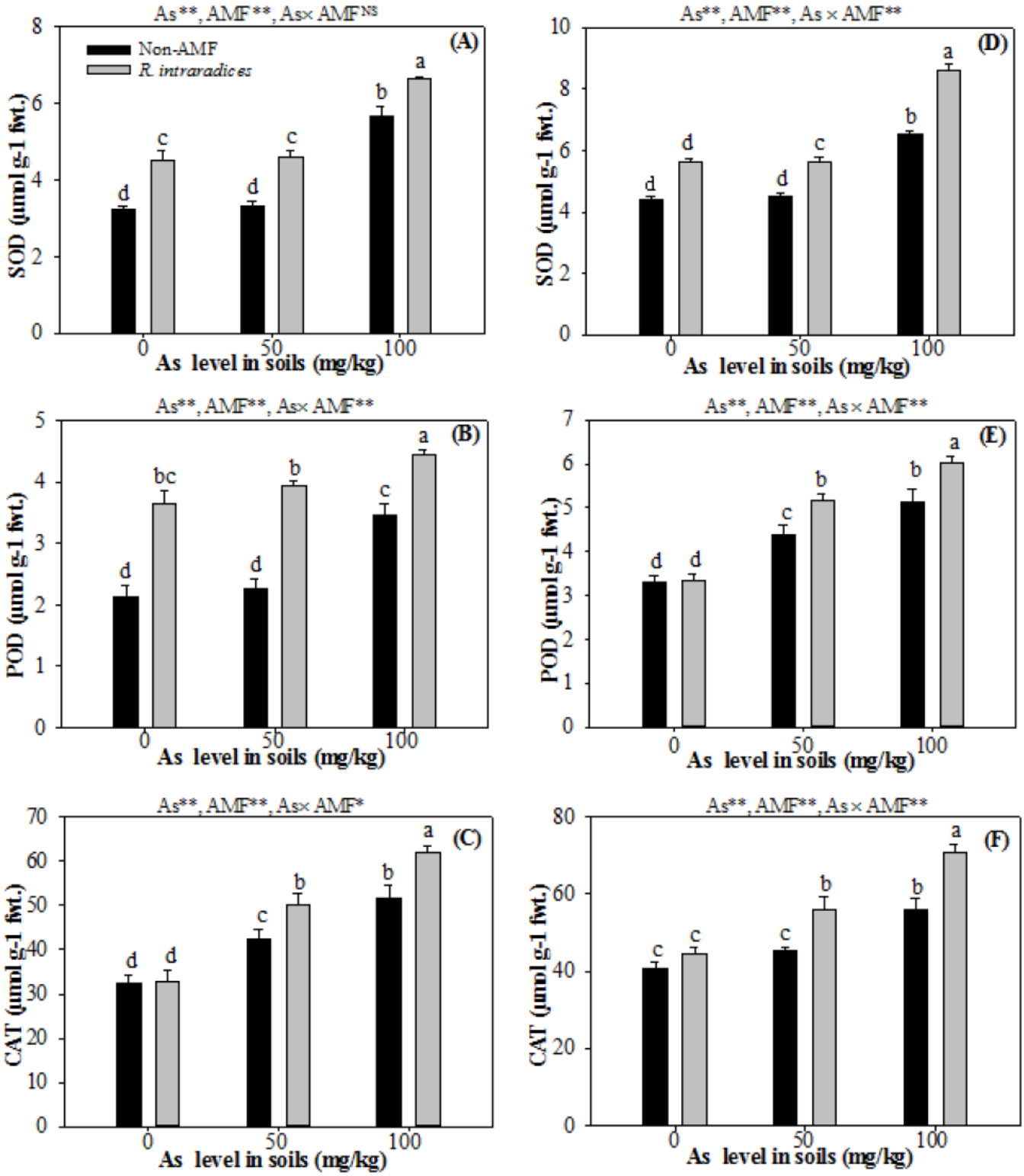


Figure 4

Effects of Rhizophagus intraradices on antioxidant enzyme in Sophora davidii leaves (A, B, C) and roots (D, E, F) under different level of As stress The same letter in each column indicates no significant difference among treatments at $P < 0.05$ using Tukey's test; Values are means ± SD, n = 5; *P < 0.05, **P < 0.01, NS not significant

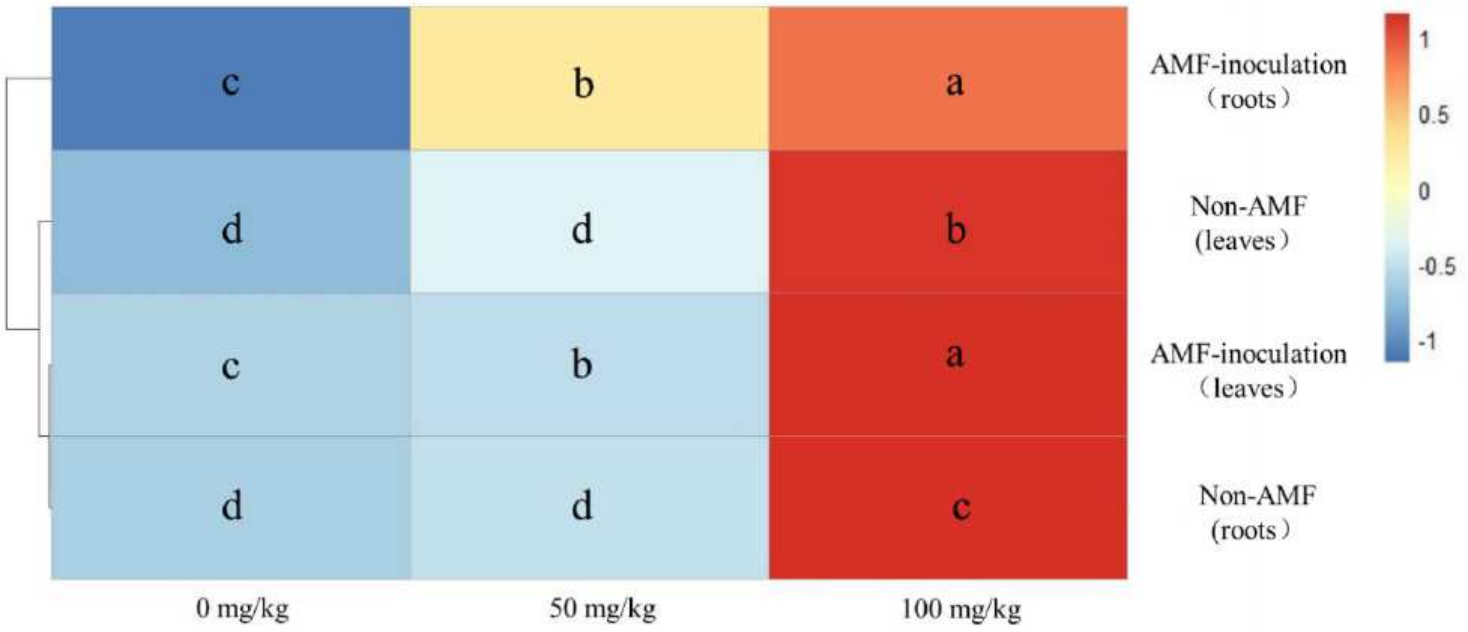


Figure 5

Effects of Rhizophagus intraradices on expression of SvPCS1 in roots and leaves of Sophora davidii. under different level of As stress Different letters within each gene indicate significant differences ($P < 0.05$).