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The Efficiency of Arbuscular Mycorrhizal Fungi in Promoting Alfalfa Growth in Acid Soils

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Abstract

High concentrations of soil Al^{3+} in acid soil severely influence the growth of *Medicago sativa* (alfalfa). The objective of the current study was to analyze whether Arbuscular Mycorrhizal Fungi (AMF) inoculation could improve alfalfa growth in acid soils. A two-way completely randomized factorial design was employed for *M. sativa* and *M. lupulina* (black medick) with two inoculations (rhizobia and AMF) and three Al^{3+} levels, and replicated four times. The soil Al^{3+} levels were adjusted to 900 mg/kg, 1000 mg/kg and 1100 mg/kg. Spores of AMF were isolated directly from rhizosphere soils of black medick. The rhizobia were isolated from root nodules in fields separately from two plant species. At each Al^{3+} level, there were four inoculations, non-inoculation, AMF solely, rhizobia solely and dual-inoculation with AMF and rhizobia. Soil Al^{3+} concentration significantly limited above- and below-ground growth of both alfalfa and black medick, reducing plant height, branching number, shoot and root weight, and root length, surface area and volume. Compared to rhizobia, AMF showed a higher tolerance to soil Al^{3+}. AMF inoculation increased the shoot and root weight of both plant species under most circumstances. Overall, AMF colonization had a trend in increasing the contents of phosphorus in both plant species at all Al^{3+} concentrations but not nitrogen and potassium. Dual inoculation significantly increased nodulation ability, enabling both plant species to form nodules at 900 and 1000 mg/kg Al^{3+}. Though the soil Al^{3+} concentration influenced the efficiency of AMF inoculation, AMF inoculation improved nodulation, increased plant growth and nutrient uptake, suggesting that it was an alternative way in improving alfalfa growth in acid soils.

Keywords: acid soil, aluminum, arbuscular mycorrhizal fungi (AMF), *Medicago lupulina*, *Medicago sativa*

1. Introduction

Medicago sativa L. (alfalfa), a perennial legume crop, is widely grown in temperate and subtropical regions all over the world for its high feeding value, good palatability, great adaptability, and high yield. With the increasing needs from dairy and beef production, alfalfa plantation area has enlarged from northern China to southern china, where about 21% of the arable land is acid soils, approximately reaching 2.03 million km^2 (Shi, Li, Xu, & Qian, 2016). However, it is generally believed that optimum alfalfa yields are associated with a soil pH in the range of 6.5 to 7.5. Soil acidity has become one of the limiting factors in planting alfalfa in the South of China (Guo, Ni, Yuan, & Huang, 2009). With the reduced pH levels in acid soils, alfalfa yield declines rapidly (Undersander et al., 1991), so do the nodulation, leaf retention, leaf to stem ratio, and crude protein content (Grewal & Williams, 2003).

Among the factors decreasing soil fertilities in acid soil, aluminum (Al) toxicity has been regarded as the main factor limiting crop yields (Foy, 1988). In neutral or alkaline soil solution, Al is present as harmless oxides and aluminosilicates (Martens, 2001). However, in soils with pH below 5.5, the solubility of aluminum increases greatly and is released into the soil solution in the form of toxic ions to plants [$Al(OH)^{2+}$, $Al(OH)^{2+}$, Al^{3+} and $Al(H_2O)_6^{3+}$] (Kochian, Piñeros, & Hoekenga, 2004; Rouphael, Cardarelli, & Colla, 2015). Increased concentrations of Al^{3+} caused damage to the root tip, leading to the inhibition of root growth, and ultimately

limiting the plants from adsorbing nutrients and water from soil solutions (Langer, Cea, Curaqueo, & Borie, 2009). For example, high concentrations of Al^{3+} reduced the absorption of Ca^{2+}, K^{+}, PO_4^{3-} and other essential nutrient elements by crops (Talor, 1988). Al^{3+} reduced the availability of inorganic phosphorus by inducing adsorption precipitation reaction in the rhizosphere, and then inhibited the phosphorus into plant roots and transportation aboveground (Cumming & Ning, 2003; Macklon, Lumsdon, & Sim, 1994). Al^{3+} also could be combined with the phosphate-based DNA, inhibiting DNA replication and biosynthesis, thereby affecting the cells mitosis, and consequently reducing plant performance (Clarkson, 1985). For legume crops, higher concentrations of Al^{3+} in acidic soil also inhibited the growth of rhizobia, reduced the affinity between rhizobia and root system, and limited the biological nitrogen fixation (Whelan & Alexander, 1986).

In order to alleviate the aluminum toxicity and to improve crop yield in acid soils, alkaline ameliorators, such as lime, have been applied to neutralize acidity and increase nutrient availabilities (Crusciol et al., 2016). Lime application will increase soil pH and concentrations of Ca^{2+} and Mg^{2+}, decrease concentrations of Al^{3+} and Mn^{2+} ions, and thus improve the soil phosphorus nutrition and crop yield (Cai, Xiao, & Li, 2010). Lime application improved the soil microbial carbon, nitrogen, respiration rate and metabolic quotient (qCO_2) (M. Stenberg, B. Stenberg, & Rydberg, 2000), increased the number and diversity of rhizobia (Denton, Coventry, Bellotti, & Howieson, 2000) and the activity of antioxidant enzymes (POD, SOD and CAT) in plants (Xiao, Yang, Xiao, & Xie, 2003). However, surface lime application mainly affected the soil layer to depths of only 5cm, and inappropriate lime application rates or timing might cause imbalances of soil Ca^{2+}, K^{+} and Mg^{2+}, resulting in low yields (Walker, 2002). Further, the efficiency of lime application was also associated with other factors such as rainfall distribution, soil texture, structure, hydraulic conductivity, fauna, and crop rotation and management (Edmeades & Ridley, 2003).

Besides lime application, selecting Al tolerant crop genotypes is the most fundamental method in relieving the negative effects of acid soil on plants (Choudhary, Singh, & Iquebal, 2011). Tolerance to Al was observed in some plant species and varied among genotypes (Castilhos et al., 2011; Choudhary et al., 2011; Jan, 1991). On molecular level, genes controlling Al^{3+} resistance had also been cloned from wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), sorghum (*Sorghum bicolour* (L.) Moench) and rice (*Oryza sativa* L.) (Ryan & Delhaize, 2010). However, the long and complex process of breeding Al resistant variety makes this still impractical today (Nawrot et al., 2001; Seguel et al., 2013). For alfalfa, no Al tolerant cultivars has been reported though many studies were conducted in screening Al tolerance in alfalfa cultivars (Langer et al., 2009; Khu, Reyno, Brummer, & Monteros, 2012; Pan, Zhu, & Cheng, 2008).

Legume crops form two types of symbiosis, nodule (with rhizobium) and mycorrhizae (with arbuscular mycorrhizal fungus, AMF). Though the rhizobia have been shown to be sensitive to Al^{3+}, AMF are widely distributed in acid soils and show relative tolerance to Al^{3+} (Clark, 1997; Guo et al., 2012; Fritz et al., 2010). The formation of mycorrhizae could regulate the relationship between soil aluminum, phosphorus and plant, protecting roots from the Al toxicity (Vandamme et al., 2013). AMF could improve the growth of plant in acid soils, enhance the acid resistance (Heijne, Dam, Heil, & Bobbink, 1996), and strengthen the resistance of plants to Al^{3+} (Thompson & Medve, 1984). In a pot experiment, AMF inoculation increased nodule numbers, total nodule weight, and yields of alfalfa in an acid soil with pH of 5.45 (Guo, Ni, & Huang, 2010). However, mycorrhizal fungi differ in their responses to soil pH (Cavallazzi, Filho, Stürmer, Rygiewicz, & Mendonça, 2007), and in their colonization with plant species (Orłowska, Ryszka, Jurkiewicz, & Turnau, 2005). Therefore, selecting effective AMF strains might be an alternative choice in improving alfalfa production in acid soils.

M. lupulina (black medick), belonging to the same genus with alfalfa, is widely distributed in temperate and subtropical regions and possesses the tenacious survival and reproduction ability (Yan, Chu, Wang, Li, & Tao, 2009). Yurkova, Jacobi, Gapeeva, Stepanova, and Shishova (2015) reported that the black medick cultivar-population could be characterized as an ecologically obligate mycotrophic plant under conditions of low level of available phosphorus in the soil. A long-term cover cropping with black medic showed that *Triticum aestivum* following black medic had a higher early percent root length colonized by AMF, suggesting that cover cropping with black medic was an effective method of increasing early AMF colonization (Turmel, 2007). These results suggested that AMF from black medick might be effective in low fertility soils, benefiting its wide distribution. Therefore, in the current study, we collected AMF spores from rhizosphere soils growing black medick and identified AMF strains using molecular method (Guo et al., 2012). The AMF spores were inoculated solely or dual inoculated with rhizobia in soils with three Al^{3+} levels growing black medick or alfalfa. We measured plant aboveground and underground biomass, branching number, root length, area and volume, plant nutrients, soil nutrients, AMF colonization, and nodulation, aiming to confirm whether these AMF could improve alfalfa growth and quality in acid soils.

2 Materials and Methods

2.1 Soils

The soil was a sandy yellow soil (Orthic Acrisols) with a pH of 5.34, collected from crop lands in Jigong Mountain, Beibei (29°49′N, 106°25′E), Chongqing, China. The crop lands were cropped with maize and sweet potato for at least 10 years, and no plant from *Megicago* genus was planted. The soils were collected from 0-20 cm layer, air dried, stones and plant debris removed, sieved through 2 mm mesh, and wet sterilized at 121 ^{o}C for 25 min. The soil organic carbon, total nitrogen (N), phosphorus (P) and potassium (K) were 22.83, 0.79, 0.56 and 18.33 g/kg, respectively. The concentrations of available N, P and K were 100.12, 20.16 and 112.5 mg/kg, respectively. The CEC of the soil was 8.54 cmol(+)/kg, with the concentration of Al^{3+} reaching 1100 mg/kg.

2.2 Plant Materials

The alfalfa cultivar, *Medicago sativa* cv Sanditi, was bred in France by the Royal Barenbrug Group (The Netherlands), imported to China by Barenbrug China (Beijing), and has been widely grown in subtropical areas of China with a dormancy of 5.2 (Shen et al., 2013). The seeds of black medick (*Medicago lupulina*) were directly collected from uncultivated fields located in Beibei, Chongqing. The seeds were washed clean of commercial coating (for Sanditi) and placed on wet paper in Petri dishes under dark conditions for 12 h prior to sowing.

2.3 Symbiotic Microbes

The tested rhizobia were separated from root nodules of *M. lupulina* in field and *M. sativa* which has been cultivated for four years. They were purified and propagated using YMA medium (yeast morphology agar). The inoculants were propagated using YMA medium without agar.

The tested AMF spores were directly collected from the rhizosphere soils of wild black medick, using the wet sieving and decanting methods described by Zhao et al. (2001). In total ca. 50 kg soils from ca. 200 plants were wet sieved and ca. 300 g spores and sporocarps were obtained. Since the spores isolated from soils were a mixture, the DNA of the soils was extracted and used for AMF identification followed by the method of Guo et al. (2012). In total 5 strains were identified, mainly *Glomus* species (Table 1). The isolated spores and sporocarps were mixed with autoclaved sand (2:1), reaching a spore density of 150/g inoculants. The mixture was directly used as inoculants.

2.4 Experiment Design

A two-way completely randomized factorial design was employed for each plant species with two inoculations (rhizobia and AMF) and three Al^{3+} levels, and replicated four times. The soil Al^{3+} levels were adjusted to 900 mg/kg (Al1), 1000 mg/kg (Al2) and 1100 mg/kg (Al3), by adding lime ($Ca(OH)_2$) equivalent to 1.5 g/kg, 0.75 g/kg and 0 g/kg, respectively. About 1.5 kg dry soils were put into one pot (18 cm diameter, 14 cm height) and saturated with water for 15 days before sowing.

For AMF treatment, about 5 g inoculants were applied at a depth of 8 cm of the pot before sowing. Non-AMF treatments received same amount of autoclaved sand. The rhizobia inoculant was applied 3 times, one at the day of transplanting, and the other two at next two weeks. At each application time, each pot received ca. 5×10^4 bacteria based on an assessment of the numbers of Rhizobia present in a bacterial culture by carrying out serial dilution. The application was done with watering. Non-rhizobia treatments received the same amount of autoclaved medium. Each plant species contained non-inoculated group, AMF group, Rhizobia group, and AMF+Rhizobia group, grown in soils with three different Al^{3+} levels.

Ten seedlings were transplanted in each pot and thinned to six plants per pot one week later. The pots were watered twice a week, keeping the relative soil moisture content between 50%-75%. The pots were placed in a glasshouse accepting sunlight, with temperatures ranging from 20 ºC to 25 ºC during daytime and 15 ºC to 20 ºC during night. The pot positions were adjusted once every week.

Table 1. AMF strains isolated from soils of *Medicago lupulina*

Clone No.	Accession No.	The strains which have the highest identity from NCBI	Accession No.	Similarity	Query coverage %
1	KY235383	*Glomus mosseae*	GU966531.1	99%	99%
2	KY235384	Uncultured *Glomus*	FR871390.1	99%	96%
3	KY235385	*Glomus sp*	FM876806.1	99%	100%
4	KY235386	Uncultured Glomus	JQ218217.1	96%	100%
5	KY235387	*Glomus sp*	FM876804.1	97%	100%

2.5 Measurements

2.5.1 Plant Growth Measurement

After two and a half months of growth, the plants were harvested and separated into shoots and roots, and the height and branching number were measured. The shoots were dried at 105 °C for 20 min and then at 75 °C for 48 h, and then weighed. The roots were carefully washed with distilled water in sieve, nodule number, root length, surface area and volume were recorded using a root scanner (WinRHIZO). Part of roots (ca. 0.2 g) was used for measuring AMF colonization, with the remaining roots dried at 75 °C for 48 h and weighed.

2.5.2 Plant Nutrient Analysis

Dried shoots were ground, sieved through 0.5 mm mesh, and digested in HNO_3/H_2O_2 solution. The digested solution was used to analyze total nitrogen (TN), total phosphorus (TP) and total potassium (TK). TN was measured by the Kjeldhal method, TP was determined by vanadomolybdophosphoric yellow color method, TK was measured by flame atomic absorption spectrometric method (Bao, 2005).

2.5.3 Soil pH and Concentrations of Aluminum, Available Nitrogen, Phosphorus and Potassium

Soil pH value was determined in a soil: water (1:5) solution using a pH meter. Aluminum concentration was determined by eriochrome cyanine R spectrophotometric method (Qiu, 1989). Alkali dispelled nitrogen (AN) was determined by titration method. Extraction of available P were carried out using HCl-NH_4F, and analyzed using the ammonium molybdate method (Olsen & Sommers, 1982). Available K were extracted by NH_4OAc and analyzed by flame atomic absorption spectrometric method.

2.5.4 AMF Colonization

Approximately 0.5 g of roots were cleared in 2% KOH (w/v) at 90 °C for 60 min and rinsed three times in water. The root samples were acidified in 2% HCl (v/v) for 30 min and then stained in 0.05% (w/v) trypan blue in lactoglycerol for 30 min at 90 °C. Root segments of each plant species were selected randomly and assessed for the presence or absence of AMF structures (arbuscules, vesicles and thick hyphae) using a stereomicroscope. AMF colonization were distinguished from non-mycorrhizal fungi as described by Callaway, Mahall, Wicks, Pankey, and Zabinski (2003). Colonization was expressed as frequency of mycorrhiza in the root system (F%) and intensity of the mycorrhizal colonization in the root system (M%) according to the method of Trouvelot, Kough, and Gianinazzi-Pearson (1986). In total three slides with 45 root segments in each root sample were observed under the microscope and rated according to the range of classes based on Mycorrhizal Manual (http://www2.dijon.inra.fr/mychintec/Protocole/protoframe.html). The values were put into the computer program 'Mycocalc' to calculate F, M and A.

2.6 Data Analyses

The data were the average from four replicates. Two-way ANOVA analysis was applied to analyze the effects of Al^{3+} concentrations and inoculation and their interactions on plant and soil parameters (SPSS 17.0, USA). Due to the significant interactions between Al^{3+} and inoculation, the effects of inoculation on soil and plant parameters were further analyzed using one-way ANOVA analysis at each Al^{3+} level, separately. The significance was based on the least significant difference test at $P < 0.05$. Pearson's correlation analysis was conducted to examine the relationship between soil parameters and plant parameters.

3 Results

The two-way ANOVA analysis indicated that soil Al^{3+} levels significantly influenced all parameters from both plants and soils, whereas inoculations significantly influenced shoot weight, root/shoot ratio, phosphorus concentration in plant, root length, surface area and volume from both plant species, and all soil parameters

except soil pH for *M. lupulina* (Table 2). There existed significant interactions between Al^{3+} levels and inoculations, varying between the two plant species.

Table 2. Analysis of variance of main effects (Al^{3+} concentration and inoculation) and their interactions for plant growth parameter and soil nutrients

	Medicago sativa			*Medicago lupulina*		
	Al	Inoculation (I)	Al × I	Al	Inoculation (I)	Al × I
Height	129.06***	3.44**	0.22ns	199.10***	2.09ns	1.09ns
Branching number	57.98***	4.10**	1.83ns	608.45***	1.40ns	6.10***
Shoot weight	150.20***	6.11***	0.49ns	1438.49***	10.79***	9.58***
Root weight	106.35***	9.93***	2.51**	86.74***	1.80ns	0.53ns
Root/Shoot ratio	21.30***	2.28*	3.41**	67.22***	5.52***	3.72***
Nitrogen content in plant	75.74***	6.21***	5.02***	192.13***	1.62ns	4.16***
Phosphorus content in plant	7.01***	13.10***	5.62***	7.14***	10.32***	2.51**
Potassium content in plant	12.76***	1.12ns	0.92ns	33.69***	2.21ns	4.25***
Root length	600.13***	9.78***	6.37***	1326.30***	14.46***	10.30***
Root surface area	789.25***	4.74***	31.01***	1383.70***	19.90***	13.13***
Root volume	364.61***	9.31***	23.85***	543.08***	12.76***	13.90***
Soil available nitrogen concentration	73.33***	3.43**	2.37*	13.87***	6.73***	1.58ns
Soil available phosphorus concentration	623.77***	6.39***	1.76ns	1453.17***	3.16**	1.51ns
Soil available potassium concentration	230.47***	19.12***	3.95***	285.72***	5.48***	5.65***
Soil pH	309.3***	2.74*	2.46**	428.49***	1.21ns	0.95ns
Soil available aluminum concentration	373.00***	8.68***	14.18***	708.68***	4.01**	1.67ns

Note. "***"represent significant at $P < 0.001$; "**"represent significant at $P < 0.01$; "*"represent significant at $P < 0.05$.

3.1 Plant Height and Branching Number

Overall, the plant height and branching number from both plant species decreased with increased soil Al^{3+} concentrations (Figure 1). Compared to plant height at Al1, alfalfa height reduced by 25.70% at Al2 and 68.60% at Al3 (Figure 1A), while black medick height reduced by 27.06% at Al2 and 74.62% at Al3 (Figure 1C). The average branching number of alfalfa reduced from 14.44 per pot at Al1 to 11.56 at Al2 and to 6.56 at Al3 (Figure 1B), whereas that in black medick reduced from 24.81 at Al1 to 20.5 at Al2 and to 7.0 at Al3 (Figure 1D).

AMF inoculation (solely or dual) exhibited a trend in increasing plant height and branching number of alfalfa at all three Al^{3+} levels, but no significant difference could be observed except for a significant increase in branching number at Al2. For black medick, only dual inoculation at Al3 significantly increased both height and branching number, with no significant changes of plant height at Al1 and Al2, and a decrease of branching number at Al1 and Al2 for dual inoculation.

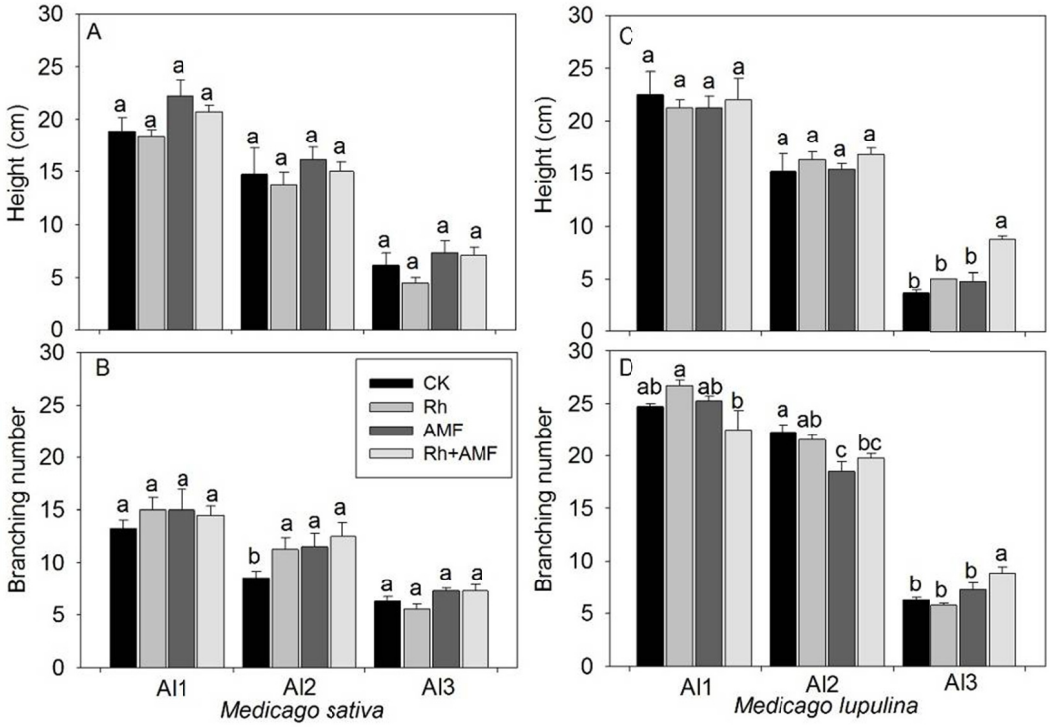

Figure 1. Effects of inoculation on height and branching number of *Medicago sativa* and *M. lupulina* grown in acid soils with different Al^{3+} concentrations

Note. Different small letters above the data bar represented significance at $P < 0.05$ (LSD). CK, Rh, AMF and Rh+AMF represented non-inoculation, rhizobia inoculation, AMF inoculation and co-inoculation with rhizobia and AMF, respectively.

3.2 Shoot Weight, Root Weight and Root to Shoot Ratio

Shoot and root weight decreased with increased Al^{3+} concentrations for both plant species (Figure 2). For example, the shoot weight at Al1 was 1.32 and 6.23 times of those at Al2 and Al3 for alfalfa (Figure 2A), and 1.54 and 9.08 times for black medick, respectively (Figure 2D). The root weight at Al1 was 1.47 and 12.03 times for alfalfa and 1.68 and 5.17 times for black medick at Al2 and Al3, respectively (Figures 2B and 2E). Due to the inconsistent responses of shoot and root weight to changes of Al^{3+} concentration, the root to shoot ratio decreased by 43.38% for alfalfa from Al1 to Al3, whereas increased by 86.67% for black medick from Al1 to Al3 (Figures 2C and 2F).

The responses of shoot and root weight to inoculations differed between the two plant species. For alfalfa, AMF inoculation and dual inoculation increased shoot and root weight at all Al^{3+} concentrations except for insignificant changes of shoot weight at Al1 and Al2 for dual inoculation and root weight at Al3 for AMF inoculation. For black medick, inoculation also had a trend in increasing shoot and root weight at Al1 and Al2. At Al3, only dual inoculation significantly increased shoot and root weight of black medick. The root to shoot ratio of alfalfa inoculated with AMF was significantly higher than those inoculated with rhizobia solely at Al1 and Al2 but not at Al3. For black medick, root to shoot ratio at all Al^{3+} concentrations remained unchanged except for a decrease at Al3 for dual inoculation.

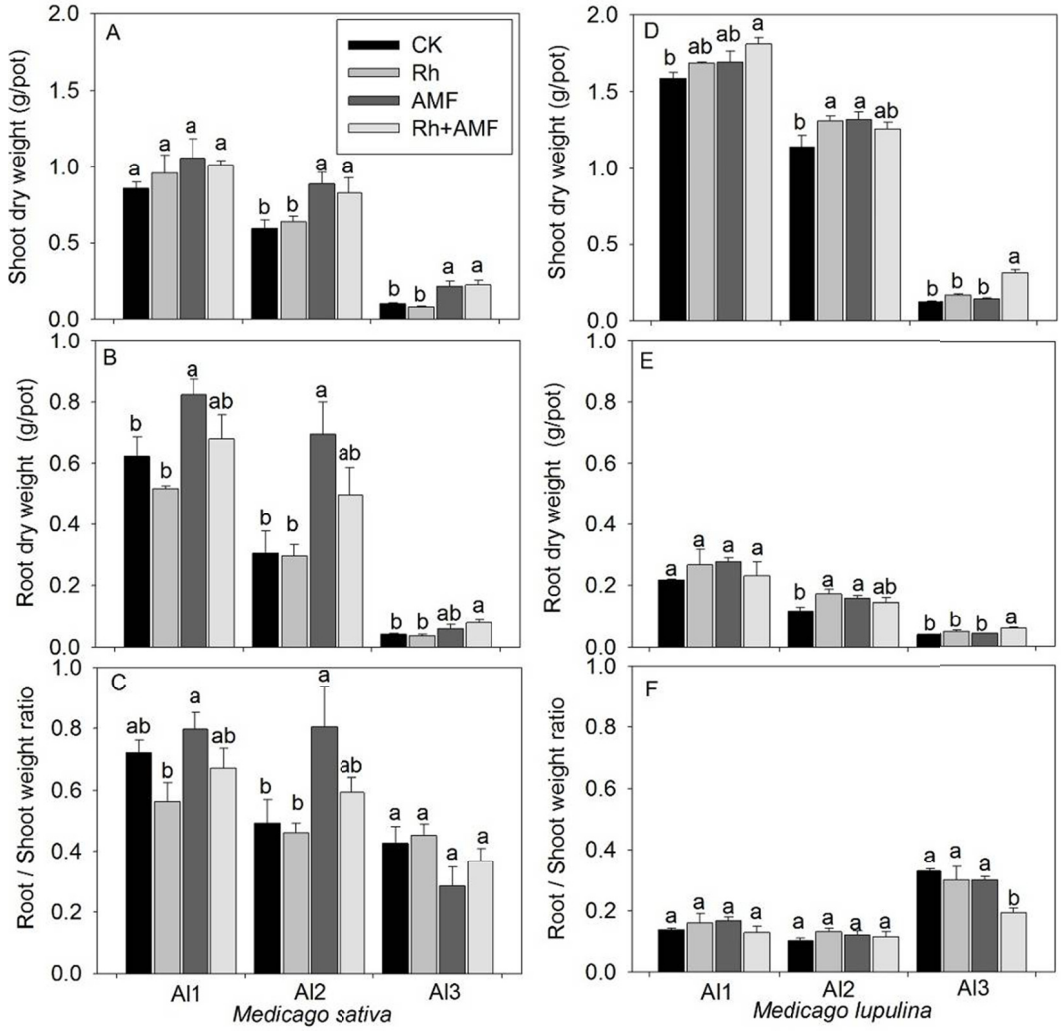

Figure 2. Effects of inoculation on shoot and root weight of *Medicago sativa* and *M. lupulina* grown in acid soils with different Al^{3+} concentrations

Note. Different small letters above the data bar represented significance at $P < 0.05$ (LSD). CK, Rh, AMF and Rh+AMF represented non-inoculation, rhizobia inoculation, AMF inoculation and co-inoculation with rhizobia and AMF, respectively.

3.3 Root Length, Root Area and Root Volume

The root length, root area and root volume of both plant species reduced with increased Al concentrations except for root length and root area of alfalfa at Al2 (Figure 3). Great decrease was observed from Al2 to Al3 for both plant species. For example, root length, root area and root volume reduced by 74.96% and 70.98%, 68.05% and 63.06%, and 60.34% and 53.59% for alfalfa and black medick, respectively, when Al^{3+} concentration increased from Al2 to Al3.

Overall, AMF inoculation had a trend in increasing root length, root area and root volume for both plant species at Al2 and Al3. At Al1, root length was unchanged whereas root area and root volume significantly reduced due to inoculation for alfalfa (Figures 3A, 3B and 3C). For black medick at Al1, root length, root area and root volume of dual inoculated plants was significantly higher than those of other treatments except for root length inoculated with rhizobia solely (Figure 3D).

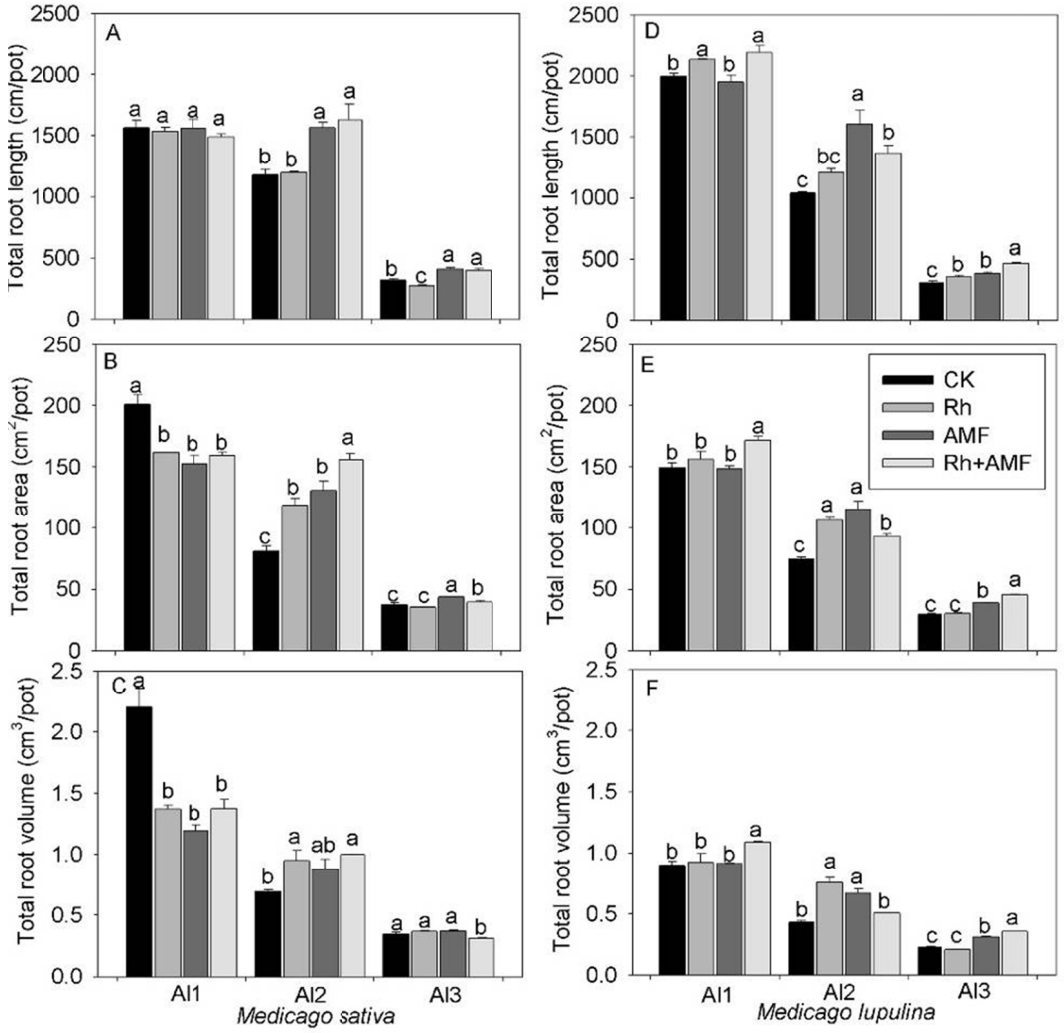

Figure 3. Effects of inoculation on root length (A, D), surface area (B, E) and volume (C, F) of *Medicago sativa* and *M. lupulina* grown in acid soils with different Al^{3+} concentrations

Note. Different small letters above the data bar represented significance at $P < 0.05$ (LSD). CK, Rh, AMF and Rh+AMF represented non-inoculation, rhizobia inoculation, AMF inoculation and co-inoculation with rhizobia and AMF, respectively.

3.4 Contents of Plant Nitrogen, Phosphorus and Potassium

Overall, concentrations of nitrogen, phosphorus and potassium in both plant species increased with increased Al^{3+} concentrations except for a decrease of phosphorus (3.33%) and potassium (9.25%) in black medick at Al3 (Figure 4). The increase rate (compared to Al1) of phosphorus and potassium contents ranged from 6.14% to 9.80% and 7.82% to 13.67%, respectively. Increase rate less than 10% was also observed for nitrogen content when soil Al increased from Al1 to Al2, whereas they increased to 36.54% in alfalfa and 43.28% in black medick when soil Al increased from Al1 to Al3.

The effects of inoculation on plant nutrients differed between plant species and among Al^{3+} concentrations. For alfalfa, contents of nitrogen and potassium unchanged after inoculation at all Al^{3+} concentrations except a decrease of nitrogen content after AMF and rhizobia inoculation solely at Al2. Phosphorus contents increased for AMF and dual inoculation at Al1 and AMF inoculation at Al3 (Figure 4B). The contents of phosphorus in plants inoculated with rhizobia was significantly lower than those inoculated with AMF and both AMF and rhizobia at Al1, those inoculated with both AMF and rhizobia at Al2, and those inoculated with AMF at Al3. For black medick, the contents of nitrogen increased after inoculation at Al2 and unchanged at the other two Al^{3+}

concentrations, the contents of phosphorus increased after inoculation at Al3 and unchanged at the other two Al^{3+} concentrations, whereas the contents of potassium unchanged at all Al^{3+} concentrations except for a lower potassium content for AMF and dual inoculation than rhizobia at Al2 (Figure 4F).

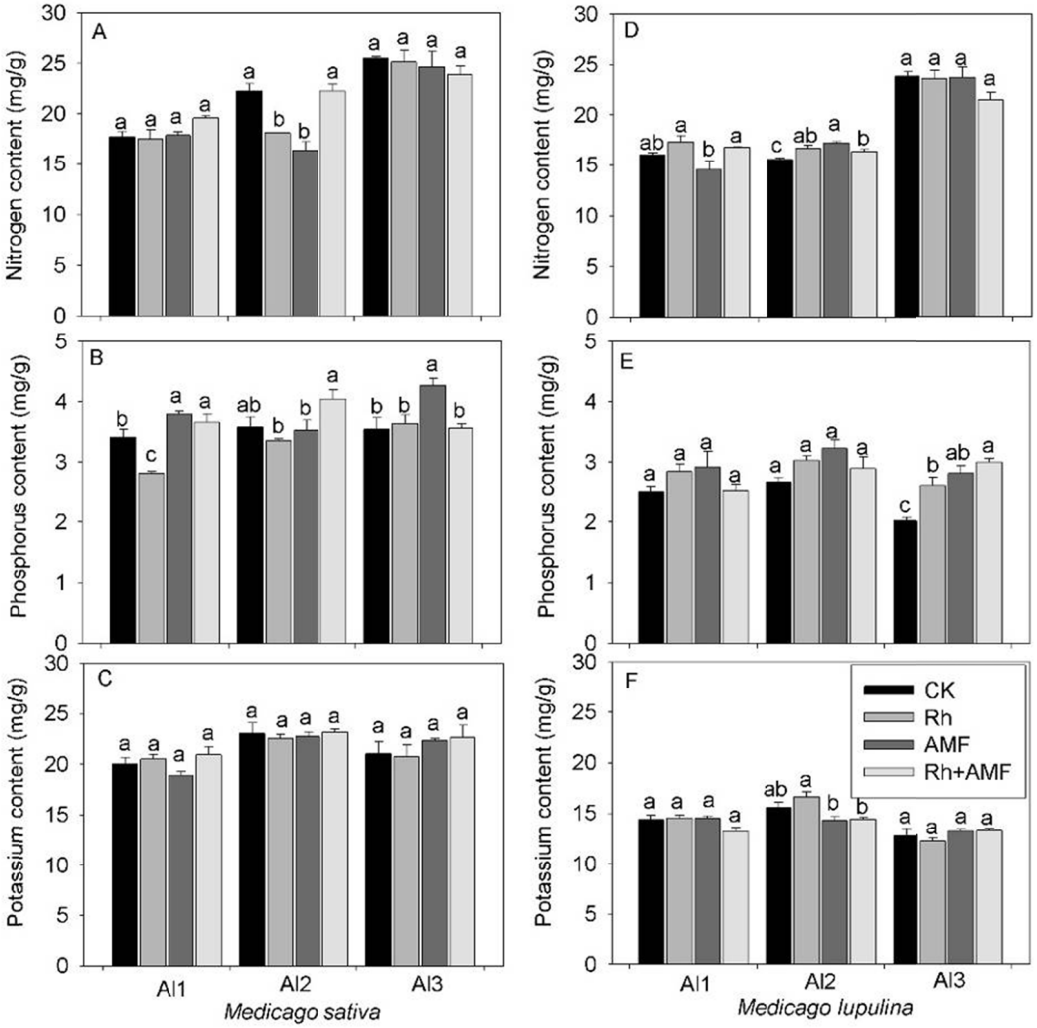

Figure 4. Effects of inoculation on contents of nitrogen (A, D), phosphorus (B, E) and potassium (C, F) in *Medicago sativa* and *M. lupulina* grown in acid soils with different Al^{3+} concentrations

Note. Different small letters above the data bar represented significance at $P < 0.05$ (LSD). CK, Rh, AMF and Rh+AMF represented non-inoculation, rhizobia inoculation, AMF inoculation and co-inoculation with rhizobia and AMF, respectively.

3.5 Soil pH and Concentrations of Aluminum, Available Nitrogen, Phosphorus and Potassium

Soils were collected after plants were harvested and analyzed for pH, concentrations of aluminum, available nitrogen, phosphorus and potassium. Compared to the initial ones before planting, soil pH changed insignificantly, whereas concentrations of Al^{3+}, available nitrogen, available phosphorus and available potassium all decreased at Al1 (Figures 5 and 6). With increased Al^{3+} levels, soil pH reduced, whereas concentrations of available nitrogen, phosphorus and potassium increased except for concentrations of available nitrogen at Al2 which unchanged from Al2 to Al3 for alfalfa (Figure 6A) and reduced from Al1 to Al2 for black medick (Figure 6D).

The effects of inoculation on soil parameters varied greatly among Al^{3+} concentrations and plant species. Soil pH unchanged for both plant species except for an increase in all inoculations for alfalfa at Al2 and a decrease in

dual inoculation at Al3 for alfalfa (Figure 5A). The concentrations of Al^{3+} decreased for alfalfa after inoculation at Al1, whereas increased when inoculated with AMF and dual inoculation at Al2 and Al3 (Figure 5B). For black medick, no significant changes were observed after inoculation at Al1 and Al3, whereas AMF and dual inoculation increased concentrations of Al^{3+} at Al2 (Figure 5D). Compared to the un-inoculated ones, inoculation with rhizobia increased the concentrations of available nitrogen at Al1, available phosphorus at Al2, and available potassium at all Al^{3+} levels for alfalfa, whereas AMF inoculation increased the concentrations of phosphorus at Al1 and Al2 and potassium at Al1 for alfalfa. For black medick, inoculation had no significant influence on the concentrations of available nitrogen, phosphorus and potassium except for a significant increase of available nitrogen for dual inoculation at Al1 (Figure 6D), and an increase of available potassium for dual inoculation at Al1 and AMF solely and dual inoculation at Al2 (Figure 6F).

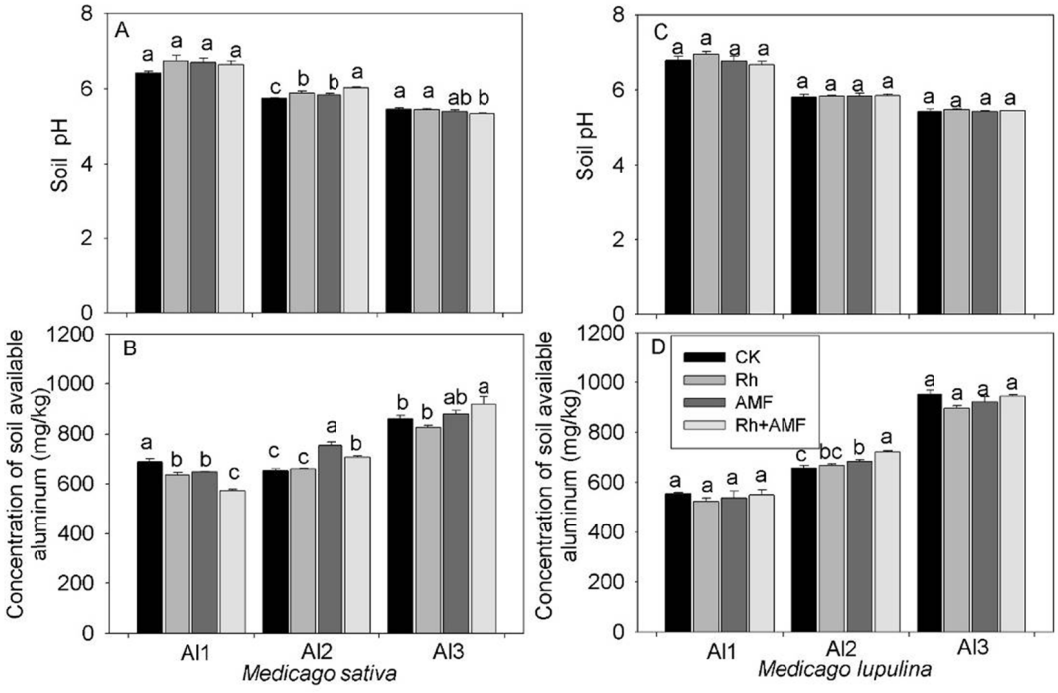

Figure 5. Effects of inoculation on soil pH (A, C) and concentrations of soil available aluminum (B, D) in *Medicago sativa* and *M. lupulina* grown in acid soils with different Al^{3+} concentrations

Note. Different small letters above the data bar represented significance at $P < 0.05$ (LSD). CK, Rh, AMF and Rh+AMF represented non-inoculation, rhizobia inoculation, AMF inoculation and co-inoculation with rhizobia and AMF, respectively.

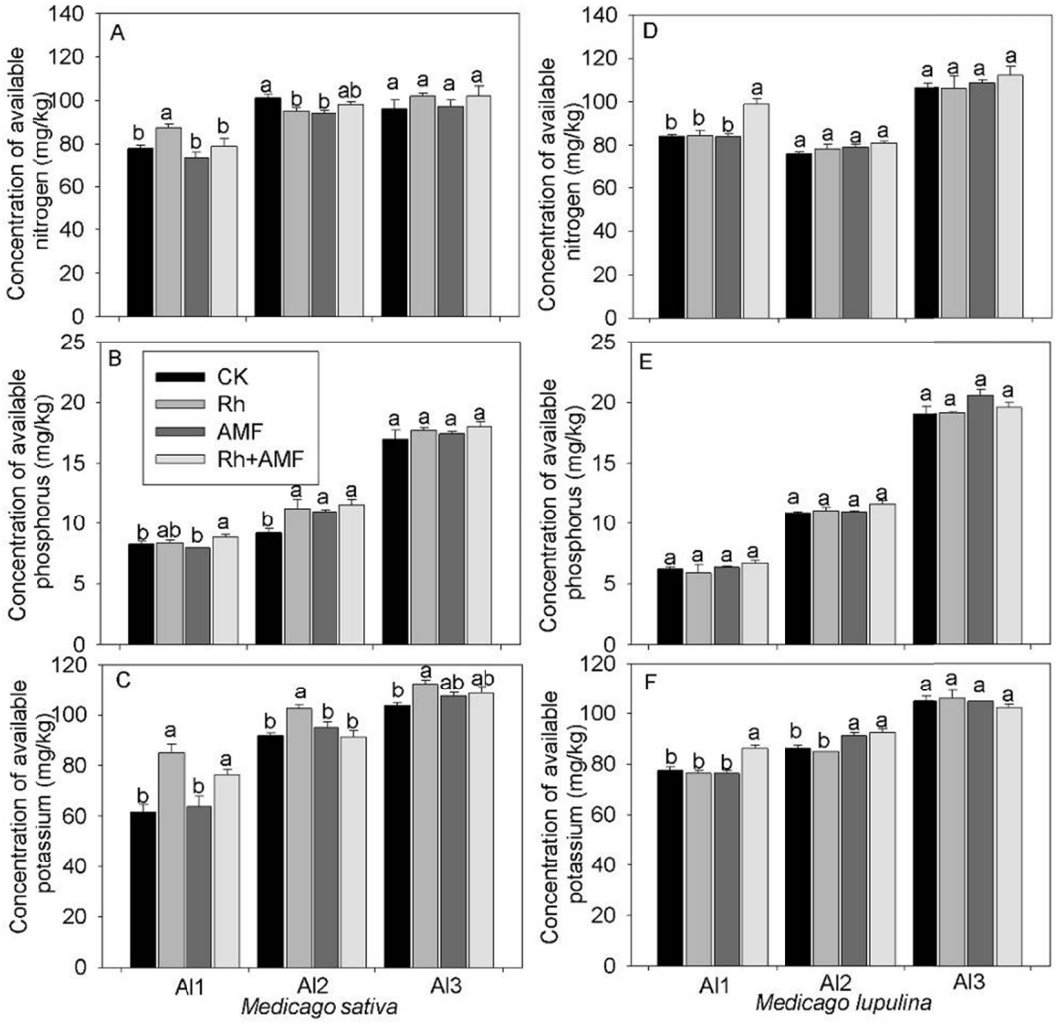

Figure 6. Effects of inoculation on concentrations of soil available nitrogen (A, D), available phosphorus (B, E) and available potassium (C, F) in *Medicago sativa* and *M. lupulina* grown in acid soils with different Al^{3+} concentrations

Note. Different small letters above the data bar represented significance at $P < 0.05$ (LSD). CK, Rh, AMF and Rh+AMF represented non-inoculation, rhizobia inoculation, AMF inoculation and co-inoculation with rhizobia and AMF, respectively.

3.6 AMF Colonization and Nodulation

Al concentrations and inoculation significantly influenced nodulations and AMF colonization except for F% in both plant species and M% in black medick, and there existed significant interactions between Al^{3+} concentration and inoculation for all parameters (Table 3). Dual inoculation significantly increased nodule number at Al1 and Al2 in both plant species. No nodule was observed at Al3. The influence of dual inoculation on AMF colonization differed among soil Al^{3+} concentrations and between plant species. For alfalfa, F% and M% increased after dual inoculation at Al1, whereas decreased at Al2 and Al3 except for an insignificant increase of F% at Al3. For black medick, dual inoculation had no significant influence on colonization except for a significant increase of M% at Al2 and decrease at Al3.

3.7 Correlation Analysis

Correlation analysis indicated that shoot and root weight were positively correlated with root length, surface area and volume, plant height, and branching number, whereas negatively correlated with contents of plant nitrogen, phosphorus and potassium except for positive relationship between root weight and phosphorus and potassium

contents (Table 4). Among the contents of plant nitrogen, phosphorus and potassium, only the nitrogen content was positively correlated with concentrations of soil Al^{3+}, nitrogen, phosphorus and potassium. Concentrations of soil Al^{3+}, nitrogen, phosphorus and potassium were all negatively correlated with shoot and root weight, root length, area and volume, plant height and branching number, whereas soil pH showed positive relationship with these parameters.

Table 3. Effects of Al^{3+} concentration and inoculation on nodule number, frequency of mycorrhiza (F%), intensity of the mycorrhizal colonization (M%) and arbuscular abundance (A%) in *Medicago sativa* (alfalfa) and *M.lupulina* (black medick) grown in acid soils

Aluminum	Inoculation	Nodule number		F%		M%		A%	
		Alfalfa	Black medick	Alfalfa	Black medick	Alfalfa	Black medick	Alfalfa	Black medick
Al1	Rh	0.00±0.00b	0.00±0.00b	-	-	-	-	-	-
	Rh+AMF	12.00±2.45a	2.25±0.63a	82.07±2.44a	74.07±4.09a	16.45±0.95a	2.51±0.88a	6.66±1.40a	0.40±0.26a
	AMF	-	-	63.54±2.78b	65.18±3.18a	12.00±1.63b	2.19±0.36a	2.07±0.33b	0.55±0.22a
Al2	Rh	2.50±0.20b	0.00±0.00b	-	-	-	-	-	-
	Rh+AMF	4.50±0.29a	2.25±0.25a	67.62±1.53b	75.56±3.57a	2.14±0.41b	14.73±1.31a	0.45±0.24b	1.21±0.03a
	AMF	-	-	87.34±0.27a	82.96±3.18a	6.02±0.78a	4.67±1.02b	2.14±0.37a	0.17±0.02b
Al3	Rh	0.00±0.00	0.00±0.00	-	-	-	-	-	-
	Rh+AMF	0.00±0.00	0.00±0.00	92.22±0.45a	87.29±2.95a	2.53±0.53b	13.16±1.8b	0.49±0.15b	5.19±0.90a
	AMF	-	-	86.67±2.72a	93.26±0.87a	16.57±3.48a	20.63±0.63a	7.31±1.51a	5.69±1.17a
Analysis of variance (F)									
Al		17.80***	11.05***	37.35***	21.64***	18.47***	86.76***	7.15***	41.35***
Inoculation (I)		32.00***	44.18***	0.81ns	0.34ns	10.83***	1.17ns	3.37*	0.06ns
Al × I		20.25***	11.05***	47.97***	4.13**	15.35***	31.63***	21.38***	0.85ns

Note. Different small letters represented significance at $P < 0.05$ (LSD). Rh, rhizobia inoculation solely; AMF, AMF inoculation solely; Rh+AMF, co-inoculation with rhizobia and AMF.

Table 4. Correlation analysis among plant and soil parameters (n = 96)

	Shoot	Root	R/S	H	Br	Rl	Ra	Rv	N	P	K	AN	AP	AK	pH
Shoot	1.000														
Root	0.389**	1.000													
R/S	-0.241*	0.757**	1.000												
H	0.873**	0.614**	0.066	1.000											
Br	0.928**	0.208*	-0.400**	0.781**	1.000										
Rl	0.905**	0.568**	0.025	0.896**	0.815**	1.000									
Ra	0.773**	0.739**	0.285**	0.859**	0.649**	0.917**	1.000								
Rv	0.510**	0.763**	0.477**	0.682**	0.366**	0.684**	0.905**	1.000							
N	-0.838**	-0.414**	0.143	-0.784**	-0.804**	-0.777**	-0.691**	-0.501**	1.000						
P	-0.249*	0.316**	0.490**	-0.031	-0.289**	-0.104	0.034	0.143	0.287**	1.000					
K	-0.244*	0.398**	0.616**	0.014	-0.331**	-0.065	0.106	0.244*	0.214*	0.735**	1.000				
AN	-0.615**	-0.450**	-0.056	-0.654**	-0.625**	-0.578**	-0.595**	-0.522**	0.658**	-0.036	-0.014	1.000			
AP	-0.876**	-0.586**	-0.075	-0.909**	-0.781**	-0.931**	-0.886**	-0.721**	0.761**	0.033	-0.055	0.686**	1.000		
AK	-0.728**	-0.646**	-0.179	-0.817**	-0.657**	-0.764**	-0.822**	-0.773**	0.666**	0.034	0.071	0.719**	0.820**	1.000	
pH	0.832**	0.580**	0.073	0.852**	0.726**	0.859**	0.864**	0.719**	-0.660**	-0.147	-0.095	-0.577**	-0.868**	-0.818**	1.000
Al	-0.881**	-0.491**	0.013	-0.870**	-0.802**	-0.895**	-0.825**	-0.634**	0.727**	0.063	-0.005	0.648**	0.945**	0.745**	-0.858**

Note. ** represent significant at $P < 0.01$; * represent significant at $P < 0.05$.

R/S, Root/shoot ratio; H, height; Br, branching number; Rl, root length; Ra, root surface area; Rv, root volume; N, nitrogen content; P; phosphorus content; K, potassium content; AN, soil available nitrogen; AP, soil available phosphorus; AK, soil available potassium; Al, soil Al^{3+}.

4. Discussion and Conclusion

Though both Al^{3+} concentrations and inoculation influenced plant growth parameters and concentrations of soil nutrients, Al^{3+} concentration had predominant effects over these parameters, showing significantly higher F value in two-way ANOVA analysis compared to that from inoculation. The effects of inoculation varied greatly among different Al^{3+} levels and showed significant interactions with Al^{3+} concentrations, suggesting that the efficiency of AMF inoculation in increasing legume yield in acid soils relying on initial soil Al^{3+} concentrations.

It is generally accepted that higher concentrations of soil Al^{3+} will result in abnormal growth of crops (Piñeros, Shaff, Manslank, Alves, & Kochian, 2005; Valle, Carrasco, Pinochet, & Calderini, 2009). In our study, plant height, branching number, shoot and root weight, and root growth all reduced with increased soil Al^{3+} concentrations. And a great decrease was observed when soil Al^{3+} concentration increased from 1000 mg/kg to 1100 mg/kg, causing ca. 80% decrease in shoot weight and ca. 90% root weight for both alfalfa and black medick. This confirmed that both plant species were sensitive to high soil Al^{3+} concentrations, attributing to the limitations of high Al^{3+} concentration to root development, and thus the aboveground growth. In solutions with 0, 2 and 4 mM $AlCl_3$, the root length inhibition rates reached ca. 80% at 4 mM Al^{3+} for alfalfa (Pan et al., 2008). In soils with 0.7 cmol/kg Al^{3+}, root weight reduced by 35% to 73% (differed among cultivars) compared to those in soils with 0.02 cmol/kg Al^{3+} (Khu et al., 2012). Root weight of alfalfa exposed to 100 μM $AlCl_3$ reduced by 56.1% than those in 0 μM $AlCl_3$ (Wang, Ren, Huang, Wang, Zhou, & An, 2016). In solutions with 2 mM $AlCl_3$ and 4 mM $AlCl_3$, 12 from 13 alfalfa cultivars showed significant inhibition of biomass accumulation when compared to that in 0 mM $AlCl_3$ (Pan et al., 2008).

Though plant growth was reduced in high Al^{3+} soils, the contents of nitrogen, phosphorus and potassium in alfalfa and medick showed an increase trend with increased Al^{3+} concentrations in the current study. For example, the nitrogen content increased 36.56% and 43.27% in alfalfa and black medick, respectively, when soil Al^{3+} concentration increased from 900 mg/kg to 1100 mg/kg. Such increase, on one hand, implied that aluminum in soil stimulated nutrients uptake by alfalfa and black medick. Osaki, Watanabe and Tadano (1997) reported that the nitrogen, phosphorus and potassium in Al-tolerant plant species such as *Melastoma malabathricum*, *Melaleuca cajuputi*, *Acacia mangium*, *Hydrangea macrophyila*, *Vaccinium macrocarpon*, *Polygonum sachalinense*, and *Oryza sativa*, were stimulated by application of Al, whereas inhibited in Al-tolerant plant species such as *Hordeum vulgare*. Concentrations of nitrogen and potassium in leaves of montane forest tree seedlings increased significantly with increasing Al^{3+} concentrations (Rehmus, Bigalke, Valarezo, Castillo, & Wilcke, 2015). Since legumes, such as alfalfa and black medick, are not regarded as Al tolerant species, other factors might be involved in regulating nutrients uptake from Al^{3+} rich soils. In this study, overall, an increase of soil available nitrogen, phosphorus and potassium were also observed with increased Al^{3+} concentrations. And the plant nitrogen content was positively correlated with the concentrations of soil Al^{3+}, available nitrogen, phosphorus and potassium.

On the other hand, the Al^{3+} concentrations in this study were adjusted by adding lime. Lime application reduced soil Al^{3+} concentration and increased soil pH value whereas reduced the concentrations of soil available nitrogen. This was inconsistent with the results from most studies where lime was applied to improve soil nutrient availabilities (Barman, Shukla, Datta, & Rattan, 2014; Brown, Koenig, Huggins, Harsh, & Rossi, 2008; Sova, 1996). One possible reason for this inconsistency of the increase of plant nutrients and the decrease of plant growth with increased Al^{3+} concentrations might be related to the plant morphology or nutrient dilution effects (Jarrell & Beverly, 1981). The plant growth was severely limited at 1100 mg/kg Al^{3+}, where more leaves rather than stems were relatively formed for small plants, resulting in relatively higher amounts of nitrogen content (He et al., 2015).

Though the efficiency of inoculation in improving plant growth was not as great as adjusting Al^{3+} concentrations did in acid soils, AMF inoculation did increase the shoot weight and root weight of both plant species under most circumstances, suggesting that it was also an alternative way in improving alfalfa growth in acid soils. This was consistent with the results from other similar studies (Guo, Ni, & Huang, 2010; Yano & Takaki, 2005). However, unlike the single strain or mix of certain AMF inoculant used in these studies, the AMF spores used in the current study were isolated directly from soils growing black medick. In total five AMF strains, mainly *Glomus* species, were observed, suggesting that the AMF strains might have displayed synergistic or competitive behavior to increase colonization. The effectiveness of mycorrhizal colonization varied between the fungal isolates introduced (Orłowska, Ryszka, Jurkiewicz, & Turnau, 2005). At a grassland mine restoration site, the use of local soil as an inoculum had greater effects on native and non-native plants than the commercial AMF inoculum used (Emam, 2016). However, it is difficult to determine the relative contribution of each AMF group to the colonization without the use of real-time PCR

methods (Alkan, Gadkar, Yarden, & Kapulnik, 2006; Jin, Germida, & Walley, 2013). Anyway, the AMF spores isolated from black medick had high compatibility with alfalfa, showing high colonization and could be applied on alfalfa.

The effects of inoculation on root characteristics differed greatly among soil Al^{3+} concentrations and between plant species. For black medick, AMF inoculation improved root growth, benefiting their adaptations to acid soils. For alfalfa, AMF inoculation overall increased root growth at Al2 and Al3 but decreased at Al1. This was consistent with the changes of AMF colonization. A significant lower AMF colonization was also observed at Al1 compared to Al2 and Al3 for alfalfa, suggesting that higher colonization might benefit alfalfa when growing in acid soils. Paudel, Baer and Battaglia (2014) reported that a higher degree of AMF colonization, relative to native co-occurring species, might partly explain the successful invasion of *Triadica sebifera* into coastal plant communities of the southeastern USA. However, a significant lower shoot and root weight at Al3 compared to Al1 and Al2 implied that AMF colonization might be not related to dry matter yield, mainly related to the difference of dominant AMF strains at different soil Al^{3+} concentrations. Clark (1997) found that maximum enhancement of plant growth in acid soil varied with AMF isolate and soil pH, indicating adaptation of AM isolates to edaphic conditions. Long-term lime application changed soil nutrient availability and increased AMF colonization, but decreased AMF phylotype diversity, implying that soil chemical properties may determine the distribution of AMF in acid soils (Guo et al., 2012). The higher AMF colonization at Al3 compared to Al1 might also be related to the soil available phosphorus concentrations. Soil available phosphorus concentrations increased with increased Al^{3+} concentrations, thus influencing the AMF colonization. Zhang, Wang, Ma, Zhang and Fu (2016) also reported that greater colonization of roots by AMF was possibly achieved with inoculating AMF isolate in soils with high P availability.

The main function of AMF colonization has been shown to be their effects on nutrient uptake from infertile soils (Smith & Read, 1997). In the current study, the AMF inoculation had more influence on phosphorus content rather than nitrogen and potassium contents. Overall, AMF colonization and dual colonization with rhizobia had a trend in increasing the contents of phosphorus in both plant species at all Al^{3+} concentrations. This was very important for these legumes growing in acid soils where soil phosphorus was generally a limiting factors (Tchienkoua & Zech, 2010), mainly attributing to the enlargement of the phosphorus adsorbing surface of the plant by the AMF hyphae (Li, Marschner, & George, 1991). Another important role of AMF inoculation in acid soils might be in conferring Al resistance to their host plants through Al-P interactions (Seguel, Cumming, Klugh-Stewart, Cornejo, & Borie, 2013), increasing plant tolerance to high levels of Al, and thereby improving nutrient acquisition (Lux & Cumming, 2001). In the current study, higher AMF colonization was observed at all Al^{3+} levels, showing high Al tolerance for these isolated AMF spores. Furthermore, AMF inoculation also influenced the concentrations of soil Al^{3+}, available nitrogen, phosphorus and potassium. However, no consistency could be observed between two plant species and among Al^{3+} concentrations except that AMF solely and dual inoculation increased soil Al^{3+} for two plant species at Al2. This implied that AMF colonization improved the plant growth in acid soils mainly through enlarging nutrient adsorbing area but not by altering the availability of soil nutrients.

Formations of nodules are very important for legumes, particularly in low fertility soils. In the current study, no nodule was observed in two plant species at all Al^{3+} concentrations except for alfalfa at Al2, when rhizobia was inoculated solely. This implied that factors other than soil Al^{3+} and pH might also limit the nodulation, particularly minerals such as Mo and Co (Rosolem & Caires, 2000; Leite, Araújo, Costa, & Ribeiro, 2009), the availabilities of which might also be limited in acid soils or influenced by lime application (Mandai, Pal, & Mandai, 1998). However, dual inoculation significantly increased nodulation ability and both plant species formed nodule at Al1 and Al2, suggesting that the formation of mycorrhizae by AMF improved nodulation for legumes growing in acid soils. This might also be attributed to the improvement of mineral adsorptions by AMF hyphae in acid soils. Meanwhile, dual inoculation increased AMF colonization (the intensity of the mycorrhizal colonization, M%) in alfalfa at Al1, reduced in alfalfa but increased in black medick at Al2, and reduced for both plant species at Al3. This implied that rhizobia and AMF might have competition when colonizing plant roots together. In a root-split experiment with alfalfa, Callaway et al. (2003) reported that nodulation systemically influenced AMF root colonization. The variations in AMF colonization when co-inoculated with rhizobia under different Al^{3+} concentrations might also be attributed to the difference of dominant AMF strains colonized at different soil Al^{3+} concentrations as discussed above.

5. Conclusion

Soil Al^{3+} concentrations significantly limited plant above- and below-ground growth for both alfalfa and black medick. Though the efficiency of AMF inoculation in increasing plant growth was less than that of adjusting Al^{3+} concentrations, AMF inoculation increased the shoot and root weight of both plant species under most circumstances and improved nodulation ability, suggesting that it was also an alternative way in improving alfalfa growth in acid soils. However, soil Al^{3+} concentrations influenced the efficiency of AMF in promoting alfalfa growth in acid soils. The AMF inoculant used in current study was a mixture from several strains. Further study is needed to clarify the functions of each AMF strain in improving alfalfa growth in high Al^{3+} acid soils.

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