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Effect of Phosphorus Application and Arbuscular Mycorrhizal Fungi Inoculation on the Growth of American Jointvetch and Greenleaf Desmodium

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Abstract: This study examined the growth and Arbuscular Mycorrhizal (AM) colonization of two tropical forage legumes, namely, American jointvetch (Aj) and Greenleaf desmodium (Gd), at two phosphate application rates (0 or 10 g P m^{-2} yr^{-1}; -P or +P), with or without AM (+AM or -AM) in a pot experiment. AM inoculation and P application promoted the growth of both species. AM inoculation in the early growth stages promoted colonization in both species, but P application did not. Nitrogen and phosphorus concentrations were affected by neither AM inoculation nor P application. Nitrogen uptake in both Aj and Gd, however, was affected by both AM inoculation and P application. Phosphorus uptake was affected by AM inoculation in Aj and by P application in Gd. The results suggest that both P application and AM promoted legume growth and AM colonization was not suppressed by P application. Nevertheless, plant responses to the treatments varied with species and growth stage.

Keywords: Arbuscular Mycorrhizal Fungi, Inoculation, Phosphorus, Tropical Legume

Introduction

Arbuscular Mycorrhizal (AM) fungi form symbiotic associations with more than 80% of land plant families. They benefit their host principally by increasing the uptake of relatively immobile phosphate ions and other mineral nutrients. AM produce extraradical mycelia that grow beyond the phosphate depletion zone around the root (Smith *et al*., 2003; Cardoso and Kuyper, 2006; Gosling *et al*., 2006; Briske, 2007; Smith and Read, 2008; Naher *et al*., 2013; Watts-Williams and Cavagnaro, 2014). Other benefits to the host include improved resistance to foliar-feeding insects, drought and soil pathogens; increased salt and heavy metal tolerance; enhanced uptake of macro- and micronutrients; and changes to soil structure (Smith *et al*., 2003; Cardoso and Kuyper, 2006; Gosling *et al*., 2006; Briske, 2007; Smith and Read, 2008; Watts-Williams and Cavagnaro, 2014). Due to their ability to increase plant nutrient uptake, AM have important roles in sustainable agriculture and natural ecosystems (Gianinazzi *et al*., 2010; Watts-Williams and Cavagnaro, 2014). Previous studies have reported that the benefits of AM are affected by soil characteristics, plant species, fertilization, climate and other factors (Cardoso and Kuyper, 2006; Kanno *et al*., 2006; An *et al*., 2008; Posada *et al*., 2008; Smith and Read, 2008). When phosphorus is abundant in the soil, the symbiosis between AM and the host plant is less evident and the fungi become parasitic (Gosling *et al*., 2006; Naher *et al*., 2013).

Along with nitrogen and potassium, phosphorus is one of the important nutrients for plant growth (Barker and Collins, 2003). Phosphorus participates in starch synthesis and degradation and nutrient transport from the soil through the roots to the shoot (Snyder and Leep, 2007). In plants, it occurs in proteins, nucleic acids, Adenosine Triphosphate (ATP), lipids, esters and enzymes and is involved in stored energy utilization, root formation and nutrient supply (particularly nitrogen and sulfur) (Whitehead, 2000; Barker and Collins, 2003; Snyder and Leep, 2007). It also plays key roles in legume root nodulation and nitrogen fixation (Graham and Carroll, 2003; Labidi *et al*., 2015). Generally, forage plants respond to phosphorus fertilization, which increases yields of Dry Matter (DM), crude protein, digestible DM and total digestible nutrients (Whitehead, 2000; Snyder and Leep, 2007). Nevertheless, plant cannot absorb metals or phosphorus from volcanic ash or acidic soils. Phosphorus forms strong bonds with metal cations like iron or aluminum in the soil (Whitehead, 2000; Barker and Collins, 2003). In acidic soil, not all of the fertilizer supplied is utilized by the plant and hence it cannot enhance growth (Whitehead, 2000). The phosphorus fertilizer is mainly provided in the form of apatite, a non-renewable phosphate mineral (Whitehead, 2000; Blanco, 2011). The depletion of this ore has prompted the reevaluation of utilizing this resource and the exploration of other phosphorus fertilizer supplies elsewhere in the world (Blanco, 2011).

The Fabaceae, or legume family, has about 600 genera and 12,000 species (Mitchell and Nelson, 2003). In the livestock production system based on grassland, soil nitrogen is the main limiting nutrient and an intensive production system requires nitrogen fertilization. Much energy is needed to make chemical nitrogen fertilizer. In addition, its constant application causes the release of nitrate by eluviation and nitrogen oxides by volatilization from the soil. Consequently, in recent years there has been a renewed interest in biological nitrogen fixation by forage legumes (Saia *et al*., 2016). These crops are very important in many tropical and subtropical farming systems. They fix atmospheric nitrogen, enhance the value of animal feed and help stabilize the soil by reducing erosion and runoff (Humphreys, 1995). Forage legumes, therefore, are key components of livestock rations both in the form of grazed pasture and harvested hay or silage (Sheaffer and Evers, 2007). They also improve soil and provide nitrogen for other crops and food both for wildlife and humans (Zemenchik *et al*., 1996; Sheaffer and Evers, 2007). American jointvetch (*Aeschynomene americana* L.) is an erect-ascending, shrub-like tropical legume. It is an annual or a short-lived perennial (Bishop *et al*., 1985; Skerman *et al*., 1988; Cook *et al*., 2005). It has a high tolerance to wet conditions (Bishop *et al*., 1985; Skerman *et al*., 1988; Tobisa *et al*., 2014) and produces large amounts of DM (Tobisa *et al*., 2005). It is used for grazing and cut-and-carry systems (Skerman *et al*., 1988; Cook *et al*., 2005) and has been introduced to the upland paddy fields of southwestern Japan (Tobisa *et al*., 2005; 2014). Greenleaf desmodium (*Desmodium intortum* (Mill.) Urb.) is a large trailing and climbing perennial tropical legume (Skerman *et al*., 1988; Cook *et al*., 2005). It is used for long-term and irrigated pastures, hay and silage and cut-and-carry systems (Skerman *et al*., 1988; Cook *et al*., 2005). Attempts have also been made to cultivate it in the subtropical grasslands of southwestern Japan (Kitamura, 1985).

There are many current studies on the combined effects of Arbuscular Mycorrhizae (AM), green manure and crop rotation on food crop and fruit tree production (Yano *et al*., 1998; Arihara and Karasawa, 2000; Kahiluoto *et al*., 2001; Cardoso and Kuyper, 2006; Gosling *et al*., 2006; Naher *et al*., 2013). In addition, many reports have investigated the functional ecology of temperate forage plants on grassland (Frey and Schüepp, 1992; van der Heijden *et al*., 1998; Hartnett and Wilson, 2002; Kojima *et al*., 2007; van der Heijden *et al*., 2008; Bauer *et al*., 2012; Eisenhauer *et al*., 2012; Sabais *et al*., 2012; Vinichuk *et al*., 2013; Schneider *et al*., 2015) but few have researched tropical and subtropical forage legumes (Dodd *et al*., 1990a; 1990b; Soedarjo and Habte, 1995; Boddington and Dodd, 2000; Alguacil *et al*., 2010; Johnson *et al*., 2013).

In this study, a pot experiment was conducted to determine the effects of AM inoculation and phosphorus application on the growth of two tropical forage legumes. Parameters tested included plant growth, mycorrhizal formation, phosphorus content and nitrogen content. It was hypothesized that the growth of two tropical forage legumes would be promoted by AM inoculation and that phosphorus application would increase plant growth but decrease AM colonization.

Materials and Methods

Plant Preparation

The plant species used were American jointvetch (*Aeschynomene americana* L. 'Glenn', Aj) and greenleaf desmodium (*Desmodium intortum* (Mill.) Urb. 'Greenleaf', Gd). Both of these are tropical forage legumes. Two or three seeds were sown per pot. After germination, seedlings were culled so that one plant was left in each pot to grow until the start of the experiment. All preprocessing and experiments were conducted in an experimental field at Miyazaki University (31°50′ N, 131°24′ E).

Plant Growth Medium and the Design of Experiment

Wagner pots (0.02 m^2) were filled with ~3 kg Miyazaki andosol (Sansou Company, Miyazaki, Japan) at pH 6.15, EC 0.27 dS m^{-1}, 1.1 g N kg^{-1}, 26 g C kg^{-1}, 67.5 mg P_2O_5 kg^{-1} as available P (Bray II method). Fertilizer was applied at the rate of 5 g N m^{-2} and 10 g K m^{-2}. Root nodules were collected from plants cultivated in different fields, soaked in 2% v v^{-1} NaOCl and rinsed with sterile water. Bacteria from the root nodules were suspended in sterile water and added to each pot. The following treatments were used and each treatment had four replicates: Absence or presence of AM (*Gigaspora margarita*, Central Glass Co. Ltd., Tokyo, Japan) inoculation (+AM or -AM) and absence or presence of phosphorus application (+P or -P). A randomized block design was used. Phosphorus fertilizer was applied at the rate of 10 g P m^{-2}. AM was inoculated at the rate of 770 spores pot^{-1}.

Cultivation Period and Management

Seeds were sown in paper pots on July 14, 2006 and transplanted to the Wagner pots on August 16, 2006, at which time the experiment began. Analyses were made on October 6, 2006 (51 d after the start of the experiment) and on November 6, 2006 (82 d after the start of the experiment). Plants were irrigated between 17:00 and 18:00 each evening. All other environment conditions were the same as those for plants growing in natural conditions.

Investigation Method

The following measurements were taken: Shoot length, the number of nodes on the main stem, the number of lateral stems, total leaf area, the number of root nodules and the fresh and DM weight of the seeds, leaves, stems, roots and root nodules. The DM weight was determined after the plants were dried for 72 h at 85°C.

The subterranean part samples were passed through a 2 mm mesh sieve. Residual soil attached to the roots was carefully removed under running water. The roots were sub sampled for the determination of AM colonization (internal hyphae, vesicles and arbuscules) following the methods of Giovannetti and Mosse (1980). Roots were cleaned with 10% KOH (w v^{-1}), bleached with 1-2% HCl (v v^{-1}), dyed with 0.05% trypan blue (w v^{-1}) and then scored for the presence or absence of AM under a compound microscope at 400×. The AM colonization level was calculated as follows: AM colonization (%) = number of intersections colonized (internal hyphae, vesicles and arbuscules)/total number of intersections examined ×100.

Each dried plant part was crushed until it could pass through a 1 mm sieve. After the samples were wet ashed, their nitrogen concentrations were determined by the indophenol method (Bolleter *et al*., 1961) and their phosphorus concentrations by the phosphor vanado molybdate method (Quinlan and De Sesa, 1955). Nitrogen and phosphorus uptake were calculated by the following formulae, respectively: Nitrogen uptake = Nitrogen concentration×DM weight, phosphorus uptake = Phosphorus concentration×DM weight.

Statistical Analysis

AM colonization data (internal hyphae, vesicles and arbuscules) were converted into arcsine values. For AM inoculation, P application and the interactions between them (AM×P), two-way Analysis of Variance (ANOVA) was conducted for each species. Significant differences were subjected to Tukey's HSD test using Statistica, v. 10 (Stat Soft, Tulsa, OK, USA). The least significant difference between mean values was used to identify statistical differences at $p<0.05$.

Results

Plant Growth

In the first analysis of Aj, the DM weights of the stem, leaf, shoot, root and whole plant of Aj were significantly higher in +AM than in -AM ($p<0.05$) and in +P than in -P ($p<0.05$) (Fig. 1a). No AM×P interaction was found. For Aj, the highest DM weight was found in the +AM/+P treatment ($p<0.05$). For Gd, the DM weights of the stem, leaf, shoot, root and whole plant total in Gd were not significantly different between +AM and -AM ($p>0.05$) but were significantly higher in +P than in -P ($p<0.05$) (Fig. 1b). No AM×P interaction was found. The highest DM weight in Gd was obtained for the +AM/+P treatment, followed by -AM/+P. The values for +AM/-P and -AM/-P were similar and were lowest of the values observed. For Gd, the DM weight of the whole plant in +AM/+P was significantly higher than that of -AM/-P ($p<0.05$).

In the second investigation of Aj and Gd, the DM weights of stem, leaf, shoot, root and whole plant of Aj and Gd were significantly higher in +AM than in -AM ($p<0.01$), in +P than in -P ($p<0.05$) (Fig. 1c and d). No AM × P interaction was found. DM weights decreased in the following order of treatments: +AM/+P, +AM/-P, -AM/+P, -AM/-P. The DM weight of whole plant in +AM/+P was significantly higher than that of -AM/+P and -AM/-P ($p<0.05$) and in +AM/-P was higher than that of -AM/-P ($p<0.05$). -AM/-P treatment was significantly lower value than 3 other treatments.

In the first analysis of Aj, the plant length and leaf area were significantly higher for +AM than -AM ($p<0.01$) and for +P than -P ($p<0.05$). Those decreased in the following order of treatments: +AM/+P, +AM/-P, -AM/+P, -AM/-P (Table 1). For Gd, the plant length, the leaf area and the number of root nodules were significantly higher for +P than -P ($p<0.05$). In the second analysis of Aj, the plant length, the number of the main stem nodes, the number of lateral branches, the leaf area and the number of root nodules were significantly higher for +AM than -AM ($p<0.05$). The number of lateral branches, the leaf area and the number of root nodules were significantly higher value for +P than -P ($p<0.05$, Table 1). For Gd, the plant length, the number of nodes on the main stem, the leaf area and the number of root nodules were significantly higher for +AM than -AM ($p<0.05$). The plant length and the number of nodes on the main stem were significantly higher for +P than -P ($p<0.05$).

AM Colonization

In the first analyses of both Aj and Gd, the colonization of internal hyphae, arbuscules and vesicles were significantly higher for +AM than -AM (Aj: $p<0.001$; Gd: $p<0.01$), but were not significantly different between +P and -P ($p>0.05$) (Fig. 2a-c). In the second analysis of Aj, the colonization of internal hyphae, arbuscules and vesicles were significantly higher for +AM than -AM ($p<0.05$) but were not significantly different between +P and -P ($p>0.05$) (Fig. 2d-f). For Gd, there were no significant differences in AM colonization between +AM and -AM or between +P and -P ($p>0.05$).

Table 1. Plant growth characteristics of the first analyses (51 d after the start of the experiment) and second analyses (82 d after the start of the experiment)

Species[a]	Item	Treatment[b] +AM/+P	+AM/-P	-AM/+P	-AM/-P	Effect[c] AM	P	AM×P
First investigation								
Aj	Plant length (cm)	38.6[a]	27.3[b]	25.2[b]	8.1[c]	***	***	NS
	Leaf area (cm^2 $plant^{-1}$)	111.8[a]	106.6[a]	90.5[a]	8.6[b]	**	*	*
	Nodule number ($plant^{-1}$)	73.0	26.0	28.6	5.4	NS	NS	NS
Gd	Plant length (cm)	14.5[a]	5.7[b]	12.3[ab]	5.5[b]	NS	*	NS
	Leaf area (m^2 $plant^{-1}$)	111.2[a]	15.1[b]	83.4[ab]	14.9[b]	NS	**	NS
	Nodule number ($plant^{-1}$)	57.6[a]	4.8[b]	24.3[ab]	8.7[b]	NS	*	NS
Second investigation								
Aj	Plant height (cm)	79.80[a]	92.25[a]	70.10[a]	15.47[b]	**	NS	*
	Plant length (cm)	88.63[a]	103.68[a]	75.23[a]	16.47[b]	**	NS	*
	Number of the main stem node ($plant^{-1}$)	30.00[a]	30.00[a]	24.50[b]	19.33[b]	***	NS	NS
	Number of the branching ($plant^{-1}$)	45.50[a]	29.00[a]	24.00[a]	0.67[b]	*	*	NS
	Leaf area (cm^2 $plant^{-1}$)	599.68[a]	481.66[a]	219.66[b]	4.67[b]	***	*	NS
	Nodule number ($plant^{-1}$)	567.24[a]	397.23[ab]	213.57[bc]	4.46[c]	***	*	NS
Gd	Plant height (cm)	20.95	18.10	16.68	12.63	NS	NS	NS
	Plant length (m)	32.53[a]	28.00[a]	27.63[a]	15.45[b]	*	*	NS
	Number of the main stem node ($plant^{-1}$)	24.25[a]	18.75[a]	19.50[b]	17.75[b]	*	*	NS
	Number of the branching ($plant^{-1}$)	18.25	11.00	12.00	9.75	NS	NS	NS
	Leaf area (cm^2 $plant^{-1}$)	136.99[ab]	166.78[a]	114.89[ab]	67.48[b]	*	NS	NS
	Nodule number ($plant^{-1}$)	493.85[a]	340.57[ab]	241.89[ab]	120.69[b]	*	NS	NS

a. American jointvetch, Aj; greenleaf desmodium, Gd. b. Arbuscular mycorrhizal fungi inoculation (+AM or -AM), phosphorus application (+P or -P). c. ***, **, * and NS indicate significant differences at $p<0.001$, 0.01, 0.05 and $p>0.05$, respectively

Table 2. Nitrogen and phosphorus concentration and uptake of two tropical forage legumes in the second analysis (82 d after the start of the experiment)

Species[a]	Plant part	Treatment[b] +AM/+P	+AM/–P	–AM/+P	–AM/–P	Effect[c] AM	P	AM×P
Nitrogen concentration (mg g^{-1})								
Aj	Total	16.16	13.68	13.90	12.21	NS	NS	NS
	Shoot	15.90	13.32	14.39	14.37	NS	NS	NS
	Root	16.94[a]	14.85[ab]	13.14[b]	16.95[a]	NS	NS	**
Gd	Total	18.03	16.11	17.07	13.15	NS	NS	NS
	Shoot	18.67	18.17	18.94	11.41	NS	NS	NS
	Root	16.99	13.50	14.95	14.52	NS	NS	NS
Nitrogen uptake (mg $plant^{-1}$)								
Aj	Total	281.38[a]	150.10[b]	92.74[bc]	4.06[c]	**	*	NS
	Shoot	209.23[a]	106.79[ab]	65.71[b]	1.80[b]	**	*	NS
	Root	72.15[a]	43.31[b]	27.03[bc]	3.40[c]	**	*	NS
Gd	Total	246.86[a]	154.29[ab]	156.35[ab]	56.47[b]	*	*	NS
	Shoot	159.17[a]	98.21[ab]	95.44[ab]	29.43[b]	*	*	NS
	Root	87.69[a]	56.08[ab]	60.91[ab]	27.04[b]	NS	*	NS
Phosphorus concentration (mg g^{-1})								
Aj	Total	1.65[a]	2.09[a]	1.92[a]	1.12[a]	NS	NS	*
	Shoot	1.53	2.02	1.87	1.28	NS	NS	NS
	Root	2.04	2.30	2.06	1.59	NS	NS	NS
Gd	Total	2.97[ab]	2.20[b]	3.43[a]	3.38[a]	*	NS	NS
	Shoot	2.71	2.09	3.30	2.75	NS	NS	NS
	Root	3.39[ab]	2.35[b]	3.59[a]	3.89[a]	*	NS	NS
Phosphorus uptake (mg $plant^{-1}$)								
Aj	Total	27.40[a]	22.57[a]	14.21[ab]	0.37[b]	**	NS	NS
	Shoot	18.82[a]	15.98[a]	9.43[ab]	0.15[b]	**	NS	NS
	Root	8.58[a]	6.58[a]	4.78[ab]	0.33[b]	*	NS	NS
Gd	Total	40.74	21.18	29.98	14.99	NS	NS	NS
	Shoot	23.19[a]	11.39[ab]	15.79[ab]	6.92[b]	NS	*	NS
	Root	17.55	9.79	14.19	8.07	NS	NS	NS

a. American jointvetch, Aj; greenleaf desmodium, Gd. b. Arbuscular mycorrhizal fungi inoculation (+AM or -AM), phosphorus application (+P or –P). c. ***, **, * and NS indicate significant differences at $p<0.001$, 0.01, 0.05 and $p>0.05$, respectively

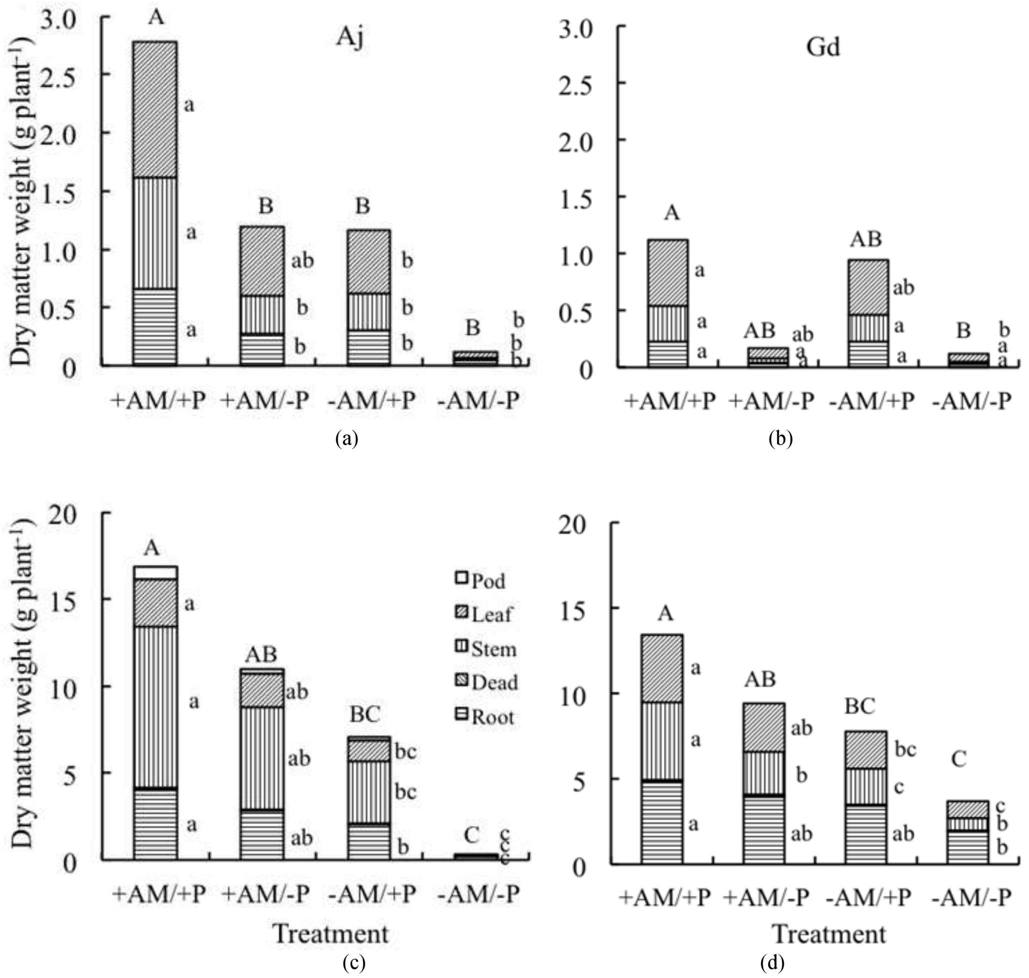
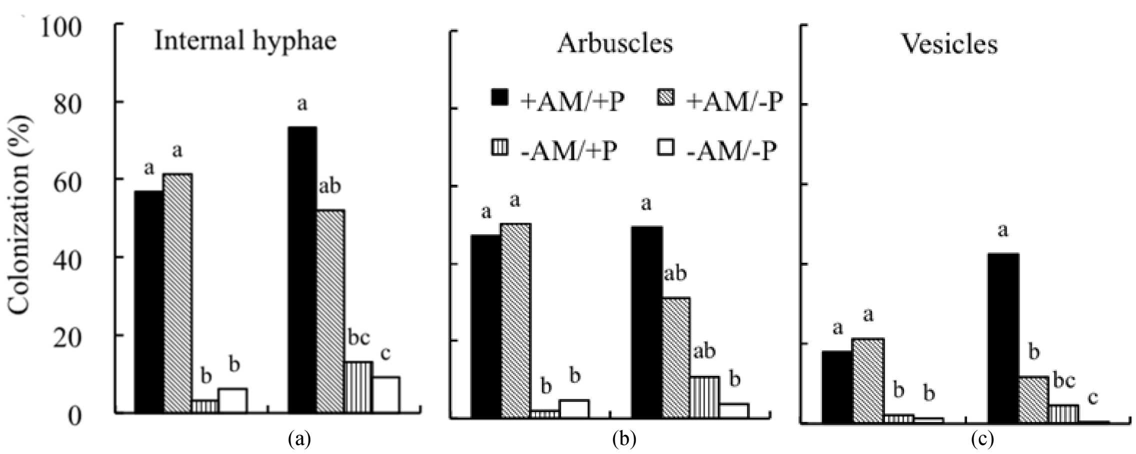


Fig. 1. Effect of phosphorous application and AM on American jointvetch (Aj) and Greenleaf desmodium (Gd). Performance at different growth stages: First analysis (51 d after experiment initiation; a and b) and second analysis (82 d after experiment initiation; c and d). Symbols with different lower case letters denote significant differences among treatments on the same date and same parts at the 5% level. Symbols with different uppercase letters denote significant differences among treatments on the same date of total weight at the 5% level. Treatment: Arbuscular mycorrhizal fungi inoculation (+AM or -AM), phosphorus application (+P or -P)



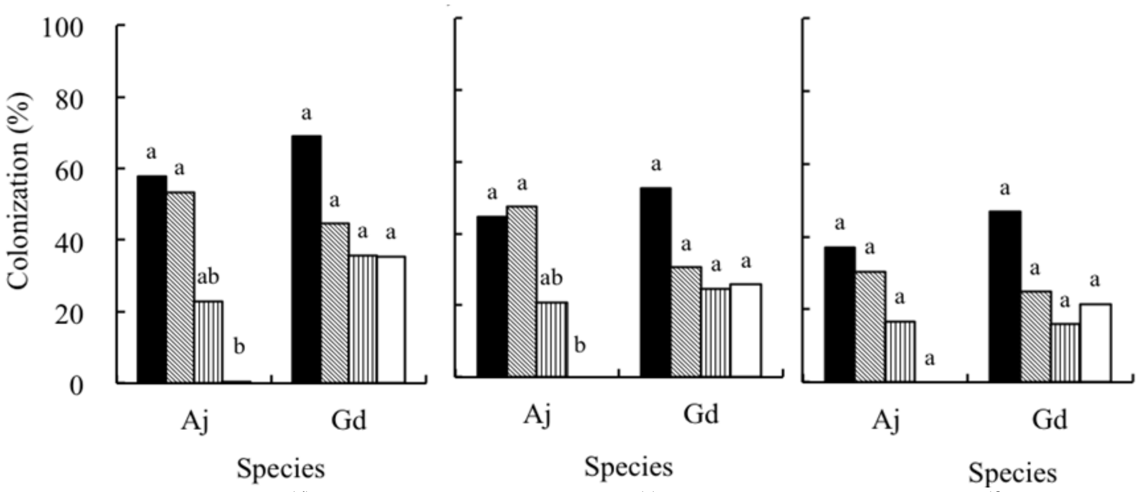


Fig. 2. Arbuscular mycorrhizal colonization (internal hyphae, a, d; arbuscules, b, e; vesicles, c, f) of first analysis (51 d after experiment initiation; a, b and c) and second analysis (82 d after experiment initiation; d, e and f). Symbols with different letters denote significant differences among treatments on the same date at the 5% level. Treatment: arbuscular mycorrhizal fungi inoculation (+AM or -AM), phosphorus application (+P or -P). Species: American jointvetch, Aj; greenleaf desmodium, Gd

Nitrogen and Phosphorus Content

For both Aj and Gd in the second analysis, there were no significant differences in the shoot, root, or whole plant nitrogen concentrations between +AM and -AM or between +P and -P ($p>0.05$, Table 2). For Aj in the second analysis, the nitrogen uptake of the shoot, root and whole plant was significantly different between +AM and -AM ($p<0.01$) and between +P and -P ($p<0.05$, Table 2). For Gd, the nitrogen uptake of the shoot and whole plant was significantly different between +AM and -AM ($p<0.05$) or between +P and -P ($p<0.05$).

In the second analysis of Aj, the phosphorus concentrations of the shoot, root and whole plant did not significantly differ between +AM and -AM or between +P and -P ($p>0.05$, Table 2). For Gd, the phosphorus concentrations of the root and whole plant were significantly different between +AM and -AM ($p<0.05$) but were not significantly different between +P and -P ($p>0.05$). In the second analysis of Aj, the phosphorus uptake of the shoot, root and whole plant were significantly different between +AM and -AM ($p<0.05$) but were not significantly different between +P and -P ($p>0.05$). For Gd, the phosphorus uptake of the shoot was significantly different between +P and -P ($p<0.05$). The phosphorus uptake of the shoot, root and whole plant were not significantly different between +AM and -AM ($p>0.05$).

Discussion

In general, when available phosphorus is abundant in the soil, or during a higher rate of phosphorus application, symbiosis between AM fungi and the host plant is decreased (Smith and Read, 2008; Naher *et al*., 2013; Yang *et al*., 2014). The rate of P application in this experiment (10 g P m^{-2}) did not suppress AM colonization in Aj and Gd Fig. 2. Therefore, it is thought that the P application rate did not influence the establishment of AM fungi in Aj and Gd. Japanese soils such as andosols are highly porous parent material of volcanic ash, have a high phosphate-fixation capacity and tend to be low in available P (Nagatsuka, 1997; ISRIC-World Soil Information, 2017). Legumes are known to have high P demands because this nutrient plays key roles in root nodulation and nitrogen fixation (Graham and Carroll, 2003; Labidi *et al*., 2015).

In the first analysis, AM inoculation promoted the growth of Aj but had no apparent effect on Gd. In the second analysis, AM inoculation promoted the growth and root nodulation of both Aj and Gd. And, AM inoculation promoted the uptake of N and P of the plant (Clark and Zeto, 2000; Vázquez *et al*., 2002; Chalk *et al*., 2006; Labidi *et al*., 2015). The results of this study corroborate those of previous reports that indicated the promotion of plant growth and nutrient uptake by AM inoculation. Growth progress data Fig. 1 and 2 indicate that AM colonization and nutrient uptake increased with plant maturation (Labidi *et al*., 2015). Nevertheless, the two species differed in terms of their acceptance of and response to, AM inoculation. Aj is an erect annual (Bishop *et al*., 1985; Skerman *et al*., 1988; Cook *et al*., 2005) whereas Gd is a prostrate perennial. Aj responded to AM inoculation and grew faster than Gd (Skerman *et al*., 1988; Cook *et al*., 2005). The two species also differed in their phosphate requirements. Aj grew poorly in -

AM/-P and probably had a higher P requirement than Gd (Skerman *et al*., 1988).

Phosphorus is essential for legume growth, root nodulation and nitrogen fixation (Barker and Collins, 2003; Graham and Carroll, 2003; Labidi *et al*., 2015). Grasses have fibrous root systems with large absorptive surface areas and compete better than legumes for P at low soil levels (Barker and Collins, 2003). AM fungi increase the uptake of N and K as well as P in plants (Sylvia *et al*., 1993; Subramanian and Charest, 1997; Naher *et al*., 2013; Saia *et al*., 2014). Phosphorus concentrations in Aj did not differ across treatments.

In the first analysis, P significantly promoted the growth of both Aj and Gd but had no apparent effect on either root nodulation or mycorrhizal formation. The application of P increased DM weight 8-9 times with AM non-inoculation and 2-7 times with AM inoculation Fig. 1. In the second analysis, P also significantly promoted Aj and Gd growth. The application of P increased DM weight by 25 and 2 times in Aj and Gd, respectively, with AM non-inoculation. Additionally, DM weight increased 1.5 and 1.4 times in Aj and Gd, respectively, with AM inoculation Fig. 1. It stimulated root nodulation but not mycorrhizal formation. P application did not increase N or P concentrations in the plants. P treatment apparently increased DM weight. Since legumes require P for root nodulation and nitrogen fixation (Barker and Collins, 2003; Graham and Carroll, 2003; Labidi *et al*., 2015), both of these were promoted by P application, thereby increasing plant DM weight and nitrogen content. AM inoculation and phosphorus application are therefore essential growth factors for both Aj and Gd. It was also shown that, based on the DM weights measured for the +AM/+P treatment, the rate of phosphorus applied in this experiment was not high enough to inhibit AM colonization.

It is assumed that there already was sufficient available P in the soil used for this experiment since for both Aj and Gd their phosphate content did not significantly differ between the +AM/-P and +AM/+P treatments. On the other hand, neither plant thrived in -AM/-P and they did not significantly grow between the first and second analyses. Both legume growth and rhizobium symbiosis can be inhibited at the early growth stages under P deficiency (Chalk *et al*., 2006; Labidi *et al*., 2015). This study indicates that Aj will not grow normally in the absence of AM at the phosphate levels occurring in the soil used for this experiment.

Finally, the results suggest that the application of both P and AM promoted the growth of two tropical forage legumes, Aj and Gd. The P application rate in this pot experiment did not suppress AM colonization. This research will provide foundational information about the cultivation and utilization of Aj and Gd in the context of AM and P in soils.

Conclusion

P application (10 g P m^{-2}) and AM inoculation promoted the growth of Aj and Gd and P application did not suppress AM colonization. Plant responses to P and AM differed according to legume species and growth stage. In future research, more detailed investigations into AM inoculation and P fertilization of each species will be necessary to optimize their management and that of grasslands in general.

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Author's Contributions

We certify that all persons who have made substantial contributions to the work reported in this manuscript.

Manabu Tobisa: He conducted field researches, made the literature review, analyzed and interpreted the results and drew conclusions and revised the manuscript and conducted the correspondence of the submitted paper.

Yoshinori Uchida: He conducted field researches, made the literature review, analyzed and interpreted the results and drew conclusions.

Ethics

The manuscript presents an original and valid work and does not infringe or violate any copy rights and neither this manuscript nor one with substantially similar content has been published or being considered for publication elsewhere.

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