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Arbuscular mycorrhizal fungi associated with tangerine (*Citrus reticulata*) in Chiang Mai province, northern Thailand, and their effects on the host plant

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ABSTRACT: There are many tangerine (*Citrus reticulata*) orchards in northern Thailand. These orchards are supplied with different levels of fertilizers. The objective of this study is to investigate arbuscular mycorrhizal (AM) fungi associated with tangerine in Chiang Mai province, northern Thailand, and the effect of AM fungi on the growth of the air layered tangerine variety 'Sai Num Phung' with different levels of nitrogen (N) and phosphorus (P) fertilizers in a pot experiment. Percentage of AM colonization in the tangerine roots and spore density in the rhizosphere varied significantly with the available P levels in the orchard soil. Means of root colonization and spore density were significantly depressed at >500 mg P/kg soil. Twenty-two species of AM fungi were found to be associated with tangerine in orchards of the Chiang Mai province. The effects of AM fungi, and N and P fertilizers on air layered tangerine plants were investigated in pots for ten months. AM fungi increased growth of the host plant especially in pots with N but without P fertilizers. AM fungi increased concentrations of P and Mg in leaves of tangerine. Application of N and P fertilizers depressed root colonization of AM fungi in the pot experiment. This study has shown that a wide range of AM fungi is associated with tangerine in commercial orchards, but with high levels of N and P fertilizers the increase in growth of tangerine trees due to the association with AM fungi may be limited.

KEYWORDS: arbuscular mycorrhizal fungi, tangerine, phosphorus

INTRODUCTION

Mycorrhizas are symbiotic associations between some soil fungi and plant roots. The host plant receives mineral nutrients while the fungus obtains photosynthetically derived carbon compounds. The most abundant association is arbuscular mycorrhiza[1]. Arbuscular mycorrhizal (AM) associations form when host roots and compatible AM fungi are both active in close proximity and the soil conditions are favourable[1]. AM fungi produce arbuscules for nutrient interchange with the host inside cortical cells and establish a diffuse network of external fine hyphae in the soil[2]. Dodd et al[3] reported that the combination treatments of AM fungi and rock phosphate have the potential to increase plant growth where phosphorus was limiting plant production. In soils with low P (phosphorus), AM fungi can enhance P uptake, plant growth, and root colonization[4]. However, Jifon et al[5] reported that inoculation with an AM fungus (*Glomus intraradices*) depressed growth of *Citrus aurantium* seedlings in soil with a high P supply. Many species of AM fungi have worldwide distribution and have apparently adapted to diverse habitats[6]. There are many tangerine (*Citrus reticulata*) orchards in northern Thailand especially in Fang District, north of Chiang Mai. A preliminary survey revealed that farmers apply fertilizers to their tangerine at different rates, from very low to very high. This is likely to affect benefits that the trees may gain from association with the AM fungi. In the mountains of northern Thailand, we identified 29 species of AM fungi in 6 genera associated with shifting cultivation[7]. In Thailand, however, information on AM association with tree crops including tangerine is limited. This study therefore set out to investigate diversity and abundance of AM fungi in tangerine orchards in Chiang Mai province, northern Thailand with different soil conditions, and to evaluate the effect of the indigenous AM fungi found in tangerine orchards in Fang on growth of the air layered tangerine variety 'Sai Num Phung'.

MATERIALS AND METHODS

Soil and root samples from the study sites

The study sites were tangerine orchards in

Chiang Mai province, northern Thailand. Forty-five soil and root samples (0–15 cm depth) were collected from the root zone of tangerine trees in 25 orchards during the rainy season (June to September) in 2005. The soil pH of the study sites ranged from 4.4 to 7.5. Most soil samples in the orchards were sandy clay loam and only four tangerine orchard soils were clay loam. The soil contained 0.90–6.87 g/kg of total nitrogen (Kjeldahl method), 28.6–817.0 mg/kg available P (Bray II method), and 20.0–724.0 mg/kg extractable K (1 N NH_4OAc, pH 7).

Evaluation of arbuscular mycorrhizal colonization in tangerine root

Root samples were washed and cut into 1–2 cm lengths. The root samples were cleared in 10% KOH at 121 °C for 15 min and washed in a sieve under running water. Cleared roots were stained with 0.05% trypan blue in lactoglycerol at 121 °C for 15 min. Thirty pieces of stained roots from each sample were mounted on glass slides to evaluate root colonization by AM fungi according to the method of McGonigle et al[8].

Determination of AM spores

AM spores were separated from 2 × 30 g of each soil sample in the root zone of tangerine by wet sieving and 50% sucrose centrifugation[1]. After centrifugation, spores in the supernatant were poured over the 40 μm sieve and washed with water to remove the sucrose before vacuum filtration on filter paper with gridlines. Spores on filter paper were kept in Petri dishes. Spores were counted under a stereomicroscope. Different types of spores were selected to observe under a compound microscope. Identification of AM fungi was done according to morphological characteristics of published AM spore descriptions[9,10].

Pot experiment for the effect of AM fungi on the host plant

The experiment was a full factorial with AM fungal inoculation (inoculated and un-inoculated treatments), two levels of N (urea at 0 and 100 mg total N/kg soil, N0 and N100), and two levels of P applied (superphosphate at 0 and 100 mg available P/kg soil, P0 and P100) with four pots per treatment. The soil used in this experiment was sandy clay loam, and had pH 6.0. The soil contained 0.41 g/kg total N (Kjedahl method), 4.1 mg/kg available P (Bray II method), 44.0 mg/kg extractable K (1 N NH_4OAc, pH 7), and 18.5 g/kg organic matter (Walkley-Black method). Each pot used in this experiment contained 15 kg sterile soil. Spores of AM fungi were isolated from indigenous soil of tangerine orchards. About 150 spores of AM fungi were inoculated in the bottom of the planting hole of inoculated treatments before planting a three feet long air layered cutting of a Sai Num Phung tangerine tree. The N and P treatments were applied in ten weekly doses. Each pot received potassium chloride at the rate 50 mg K/kg soil by application in ten weekly doses at the same time as N and P applications. The rates of N, P, and K application were chosen to support moderate growth but not to suppress infection by the AM fungi. Ten months after inoculation the leaf, stem, and root dry weights were determined. Roots from soil samples of inoculated treatments were used to determine root length colonization of AM fungi and to determine spore densities in soil samples. Leaves from each of the treatments were evaluated for N (Kjedahl method), P (dry ashing and molypdovanado-phosphoric acid), K (dry ashing, and atomic absorption spectrophotometer method), and Mg (dry ashing and atomic absorption spectrophotometer method).

Statistical analysis

Statistical tests were performed with SPSS version 10. The data were analysed by analysis of variance (ANOVA) to test the effect of the factors. Mean comparisons were determined by Waller-Duncan at $p < 0.05$.

RESULTS AND DISCUSSION

Root colonization and spore density of AM fungi association with tangerine tree

Soils in the region are generally low in available P[11]. Two thirds of samples contained > 250 mg P/kg indicating that heavy rates of P were applied in the tangerine orchards.

The wide range of available P was associated with variation in root colonization and rhizosphere spore density. Samples were grouped by cluster analysis into 3 classes by the level of available P: 29–250, 251–500, 501–817 mg P/kg soil. Mean percentage of AM colonization in the tangerine roots and spore density in the rhizosphere for each class were found to be significantly different ($p < 0.05$). Root colonization declined in soil with higher available P. With more than 500 mg P/kg soil, root colonization was only half that in soil with 250 mg P/kg or less (Fig. 1A). Spore density was not significantly different in soil with 29–500 mg P/kg, but was depressed by 85% when available soil P exceeded 500 mg P/kg soil (Fig. 1B). Many researchers showed that application of high P levels suppressed root colonization of AM fungi in

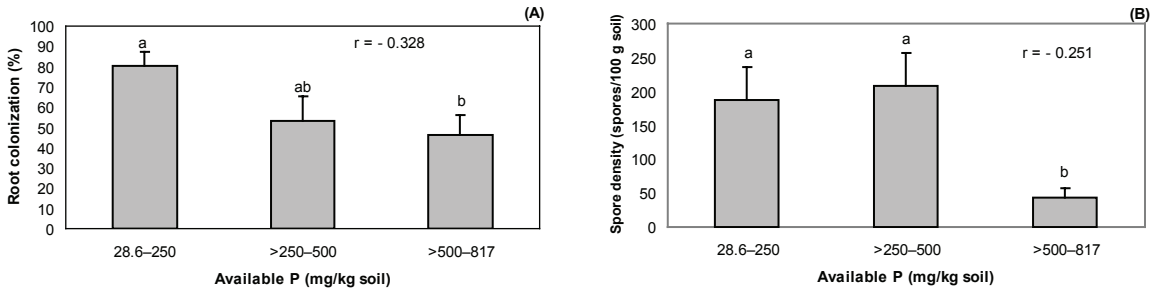

Fig. 1 Means of root colonization (A) and spore density (B) of AM fungi in different levels of available P in soils from tangerine orchards in Chiang Mai province, northernThailand during June to September 2005. Available P (mg/kg soil) 28.6–250 (16 samples), >250–500 (14 samples), >500–817 (15 samples). Columns with different letters indicate a significant difference in the means. Bars are ± standard error of the means.

host plants[12–14]. The results from this study showed a much smaller effect of P on root colonization by AM fungi than those reported. For example, Nogueira and Cardoso[14] reported that root colonization of *Gigaspora rosea* and *Glomus intraradices* in soybean decreased sharply with increasing P levels from 50 to 200 mg/kg soil. The key to this difference may be the existence of a diverse population of local AM fungi that respond differently to soil conditions. Many researchers reported that there was no relationship between spore density and root colonization[15–17]. Individual species of fungi produce spores according to their ability to proliferate in each soil condition. Thus the major depression in spore density with >500 mg P/kg soil found in this study was not matched by any depression in the percentage of root colonization.

Diversity of AM fungi in tangerine orchards

A total of 22 species of AM fungi was found in the root zone of tangerine trees in all soil samples (Table 1). Based on morphology, they were placed in the four genera, *Acaulospora***,** *Gigaspora***,** *Glomus***,** and *Scutellospora***.** All four genera were found in the three available P classes of soil samples, but the species diversity and number of species in each genus differed between the soil P classes.

In the soil samples with 28.6–250 mg/kg available P, 16 species of the AM fungi were found, in soil with more than 250 mg/kg available P there were 12 or 13 species. Some species were found in all three soil P classes. Of the individual species, *Glomus etunicatum* Becker & Gerdemann and *Acaulospora scrobiculata* Trappe were the most frequently found occurring in most samples. Some species appeared to be sensitive to high P. For example, *A. morrowiae***,** *G. invermium***,** *S. coralloidea*, and *S. nigra* were only found in soil with 250 mg P/kg or less. Diversity of the fungi in each genus also showed some variation with soil P. The number of species in *Acaulospora* declined from four in the lowest P group to two in the highest P group. In *Glomus* and *Scutellospora*, however, the number of species changed only a little with soil P, but the species themselves were different. In *Scutellospora*, the species found at 250 mg P/kg or less were entirely different from those found in soil with more than 500 mg P/kg. These results suggest that the AM fungi population depends on the available P. Bever[18] found that AM fungal species, although associating with all hosts, have host-specific differences in their population growth rates and other

Table 1 Diversity of AM fungi with different levels of available P in soils from tangerine orchards in Chiang Mai province, northern Thailand during June to September 2005.

Genera of AM fungi	Species of AM fungi in soil with available P (mg/kg soil)		
	28.6 – 250	**>250 – 500**	**>500 - 817**
Acaulospora	*A. elegans*	*A. elegans*	*A. laevis*
	A. laevis	*A. laevis*	*A. scrobiculata*
	A. morrowiae	*A. scrobiculata*	
	A. scrobiculata		
Gigaspora	*Gi. decipiens*	*Gi. decipiens*	*Gi. decipiens*
Glomus	*G. claroideum*	*G. constrictum*	*G. claroideum*
	G. etunicatum	*G. etunicatum*	*G. coremioides*
	G. hoi	*G. hoi*	*G. etunicatum*
	G. invermium	*G. macrocarpum*	*G. macrocarpum*
	G. macrocarpum	*G. mosseae*	*G. mosseae*
	G. mosseae	*G. viscosum*	*G. sinuosum*
	G. viscosum		
Scutellospora	*S. coralloidea*	*S. pellucida*	*S. aurigloba*
	S. nigra	*S. verrucosa*	*S. heterogama*
	S. verrucosa	*S. weresubiae*	*S. pellucida*
	S. weresubiae		
Total	**16 species**	**13 species**	**12 species**

components of the AM fungal community or other components of the soil community. A host variety can switch from compatible to incompatible mycorrhizal associations with a change in only one environmental variable[2].

Effect of AM fungi on the host plant in pot experiment

Arbuscular mycorrhizal fungal inoculation increased leaf, root, and total dry weight of the air layered 'Sai Num Phung' tangerine tree (Table 2). The effect of AM fungal inoculation on root dry weight of the host plants disappeared when the N and P fertilizers were applied together. Total dry weights of inoculated plants were significantly higher than those in uninoculated plants in pots with N but without P fertilizers. Hyphae of AM fungi act as a pump, supplying the root with a supplement of water and mineral salts to which it normally would not have full access[19]. This experiment found clear evidence of AM fungi influencing nutrition of the host plant. Inoculation with AM fungi generally increased the concentration of N, P, K, and Mg in tangerine leaves (Table 3). When the effect of AM on nutrient concentration was combined with the effect on leaf dry weight, the effect of AM on leaf nutrient contents was even more pronounced. Many experiments reported that AM fungi increased the P contents of the host plants. For other nutrients such as N, K, or Mg, AM fungi were reported both to have and not to have an effect on the uptake these nutrients up to host plants. It depended on the species of AM fungi and the soil conditions[20–23]. In the pot experiment, root colonization by AM fungi and rhizosphere spore density in tangerine also showed a response to N and P treatments (Table 4). Root colonization of the inoculated treatment was maximum (42.9%) in the treatment N0P0, and lowest (22.0%) in treatment of N100P100. Spore density was lowest in treatments with P100, and highest in N100P0. These values for 10 month old tangerine seedlings are much lower than that found on fully grown trees in the orchards. Nevertheless, there were similar trends in which root colonization and spore density of AM fungi were depressed by high P. In this experiment, 150 spores of mixed species of AM fungi were inoculated to air layered cuttings of 'Sai Num Phung' tangerine trees. The number of spores used for the inoculum may have been too low to penetrate the coarse roots of air layered cutting of tangerine. Clapperton and Reid[24]

Table 2 Effect of AM fungal inoculation on leaf, stem, root, and total dry weight (DW) of tangerine plants in soils with different levels of N and P, 10 months after inoculation.

Treatment	Leaf DW (g/plant)	Stem DW (g/plant)	Root DW (g/plant)	Total DW (g/plant)
N0P0M-	15.41b	51.98ab	18.87b	86.26b
N0P0M+	25.57ab	52.78ab	29.82a	108.17ab
N0P100M-	18.46b	47.31b	18.48b	84.25b
N0P100M+	24.37ab	52.58ab	31.80a	108.75ab
N100P0M-	23.70ab	50.40ab	18.59b	92.69b
N100P0M+	32.81a	61.35a	35.07a	129.23a
N100P100M-	27.80ab	61.35a	27.22ab	110.81ab
N100P100M+	30.84a	53.04ab	30.51a	114.39ab
Analysis of variance				
M	*	*	*	*
N	*	ns	ns	*
P	ns	ns	ns	ns
M×N	ns	ns	ns	ns
M×P	ns	ns	ns	*
N×P	ns	*	ns	ns
M×N×P	ns	ns	ns	ns

M+, inoculated; M-, uninoculated with AM fungi; N0, N not added; N100, 100 mg N/kg soil; P0, P not added; P100, 100 mg P/kg soil with four pots per treatment. Means in the same column followed by different letters are significantly different.
* = significant; ns = not significant.

Table 3 Effect of AM fungal inoculation on nutritional levels in leaves of tangerine in soils with different levels of N and P, 10 months after inoculation.

Treatment	Nutritional level (%)			
	N	P	K	Mg
N0P0M-	1.96c	0.10b	1.98ab	0.34c
N0P0M+	2.14c	0.13a	2.18a	0.34c
N0P100M-	1.98c	0.13a	2.15a	0.37b
N0P100M+	2.21c	0.14a	1.89bc	0.38b
N100P0M-	2.64b	0.10b	1.64c	0.37b
N100P0M+	2.73b	0.13a	1.93ab	0.39ab
N100P100M-	2.60b	0.10b	1.94ab	0.39ab
N100P100M+	3.10a	0.14a	1.88bc	0.41a
Analysis of variance				
M	*	*	ns	*
N	*	ns	*	*
P	ns	0.06	ns	ns
M×N	*	ns	ns	ns
M×P	*	ns	*	*
N×P	*	ns	ns	*
M×N×P	*	ns	ns	*

M+, inoculated; M-, un-inoculated with AM fungi; N0, N not added; N100, 100 mg N/kg soil; P0, P not added; P100, 100 mg P/kg soil with four pots per treatment. Means in the same column followed by different letters are significantly different.
* = significant; ns = not significant.

found that there was a positive correlation between the proportion of inoculum dosage and the amount of mycorrhizal colonization. Spore densities were very low in all pot experiment treatments (Table 4). The low densities of spores in this experiment may be due to the low percentage of root colonization of AM fungi. However, as mentioned above, spore density and root colonization are not necessarily closely correlated.

Table 4 Root colonization and spore density of AM fungi in soils with different levels of N and P (inoculated treatments).

Treatment	Mean of root colonization (%)	Spore density (spores/100 g soil)
N0P0M+	42.9a	17b
N0P100M+	31.9b	12c
N100P0M+	39.6ab	24a
N100P100M+	22.0c	13c
Analysis of variance		
N	*	*
P	*	*
N×P	*	ns

CONCLUSIONS

In spite of the generally accepted view that root colonization by AM fungi is depressed by high P, this study has found around 80% root colonization with up to 250 mg P/kg soil, and only slightly less with up to 500 mg P/kg soil. While it was confirmed in the pot experiment that tangerine seedlings do benefit from AM fungi association, it remains to be quantified how much the high degree of root colonization found in the field benefits the fully grown tangerine trees. The changes in species of the AM fungi at different levels of available soil P suggest that the diversity of local AM fungi population should be closely examined for better exploitation of this underground resource.

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