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Full Length Research Paper

Effects of arbuscular mycorrhizal fungi on resistance to *Phytophthora parasitica* of citrus seedlings and on growth of Thai honey tangerine scions on citrus rootstocks

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Thai honey tangerine (Sainamphueng tangerine) is generally grown by grafting on rootstocks of another variety of tangerine or citrus species which may differ in their reaction to beneficial and pathogenic soil organisms. The objectives of this study were to evaluate responses to arbuscular mycorrhizal (AM) fungi and *Phytophthora parasitica* of different citrus genotypes and the effect of AM fungi on the growth of scions of Thai honey tangerine grafted on different citrus rootstocks including Cleopatra tangerine, lime, pomelo, Swingle citrumelo and Troyer citrange. Significant differences were found among citrus species in the percentage of root colonization by the AM fungi and in the severity of root rot disease when inoculated with *P. parasitica* alone. Thai honey tangerine was most susceptible and Cleopatra tangerine was most resistant to *P. parasitica*. Inoculation with AM fungi could reduce disease severity of all the citrus plants from *P. parasitica*. AM fungi enhanced the growth of seedling to the greatest extent in lime. Variation in the response to AM fungi was found among the scions of Thai honey tangerine on different citrus rootstocks. The scion of Thai honey tangerine grew best on the lime rootstock inoculated with AM fungi.

Key words: *Phytophthora parasitica*, citrus, rootstock, arbuscular mycorrhizal (AM) fungi, root rot.

INTRODUCTION

Tangerine (*Citrus reticulata*) is grown mainly in East and Southeast Asia where it accounts for three quarters of the global planted area. Thai honey tangerine (Sainamphueng tangerine), widely grown in many parts of Thailand, is highly successful commercially but it is susceptible to root rot disease caused primarily by a soil borne pathogen, *Phytophthora parasitica*. The pathogen is capable of infecting a wide range of plants (Panabieres et al., 2005). It belongs in the kingdom Stramenopila, phylum Oomycota, and class Oomycetes (Alexopoulos et al., 1996). The oomycetes have coenocytic mycelia with mycelial walls containing cellulose and glucans. Asexual reproduction of oomycetes produces biflagellate zoospores in sporangium. Sexual reproduction of oomycetes produces oospore in oogonium (Deacon, 1997). Infection by *P. parasitica* causes brown necrosis of roots of the host plants with visible symptoms of yellow blight on the leaves that eventually die. Therefore, Thai honey tangerine is generally grown by grafting on the rootstock of another citrus variety such as Cleopatra tangerine, Swingle citrumelo and Troyer citrange.

Arbuscular mycorrhizal (AM) fungi are mutualistic associations with plant roots. They enhance mineral uptake by plant roots and reduce disease severity caused by soilborne pathogens (Trotta et al., 1996; Akkopru and

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Demir, 2005; Ozgonen and Erkilic, 2007). Mechanisms involving AM fungi in protection from plant pathogens suggested a competition with pathogens, improving plant growth and stimulating plant defense responses (Harrison, 1997; Cordier et al., 1998; Borowicz, 2001). Therefore, information on the interaction between AM fungi and the root rot pathogen on different citrus species would be valuable in the sustainable management of tangerine orchards. The objectives of this research were to evaluate the response of different citrus genotypes to AM fungi and *P. parasitica* and potential benefits of AM fungi and different citrus rootstocks for the growth of Thai honey tangerine scions.

MATERIALS AND METHODS

Effects of AM fungi on the growth and resistance to *P. parasitica* of citrus seedlings

The citrus species used in this experiment were Thai honey tangerine and Cleopatra tangerine (*C. reticulata*), lime (*C. aurantifolia*), pomelo (*C. grandis*), Swingle citrumelo (*Citrus paradisi* × *Poncirus trifoliata*) and Troyer citrange (*Citrus sinensis* × *Poncirus trifoliata*). The factorial combinations of treatments composed of six genotypes of citrus, two treatments of AM inoculation (mycorrhizal and nonmycorrhizal) and two treatments of *P. parasitica* inoculation, (inoculated and uninoculated) with four replications. Seed coat of the citrus were removed and surface sterilized in 1.2% sodium hypochlorite for 5 min, washed with sterile water twice and planted in a mixture of sterilized soil and sand (1:1, v/v) in a plastic tray. Mixture of clay loam soil and leaf litter (2:1, v/v) was sterilized at 121°C for 60 min. One month old seedlings were transplanted into each pot containing 5 kg of the sterilized soil.

For AM fungal inoculated treatment, about 400 spores of mixed species of AM fungi in soil inoculum were collected from rhizosphere of *Macaranga denticulata* in pot by wet seiving and 50% sucrose centrifugation method (Brundrett et al., 1996). They were six genera of *Acaulospora* (6 species), *Archaeospora* (1 specie), *Gigaspora* (1 species), *Glomus* (18 species), *Paraglomus* (1 species) and *Scutellospora* (3 species). AM fungal spores were placed into the planting hole, approximately 3 cm deep of soil in pot before each seedling was transplanted. Pots were placed in natural conditions in a mesh enclosure and watered once a day. Two months after AM fungal inoculation, suspension of about 10^5 zoospores of *P. parasitica* in 10 ml of sterile tap water were dip-inoculated around the root zone of each seedling.

Symptoms of citrus seedlings were evaluated one month after *P. parasitica* inoculation. The disease severity rating was based on the levels of visible symptoms that showed dulling, yellowing and browning of leaves with some eventually dropping off. The disease was rated on a scale of 0 to 5, with 0 = no visible symptom; 1 = symptom on about 20% of the leaves, through to 5 = symptom on all leaves. Soil from each pot was collected for evaluation of zoospores of *P. parasitica*. Ten grams of each soil sample was added to 90 ml of sterile water (10^{-1}), shaken vigorously for 1 min and left for 30 min to permit zoospores release from zoosporangia. Serial dilutions were then made to 10^{-4}. One (1) ml of each dilution was spread over four Petri dishes containing V8 agar and the plates were incubated at 25°C for 2 days. Colonies of *P. parasitica* were counted and the number of zoospores was calculated from colony-forming units per gram of soil.

Shoots and roots were separated and dried at 60°C for three days. The dry weight was taken to determine the effect of AM fungi on the growth of citrus seedlings. In the AM inoculated treatments, root samples were taken from each pot using 3 cm diameter soil borer at mid way between the stem and the pot wall with two soil cores per pot. Root samples from one soil core were washed and dried at 60°C to determine root dry weight in the soil cores. The dry weight of the remaining roots in the pot was also determined. The total dry weight was then calculated for all roots. Root samples from the other soil core were used to determine root colonization of AM fungi. The root samples were washed, cut into pieces about 1 cm, cleared in 10% KOH and rinsed with water on a sieve. They were then stained with 0.05% trypan blue at 121°C for 15 min (Brundrette et al., 1996). Thirty pieces of stained roots were taken and mounted on microscopic slides to determine root colonization of AM fungi (McGonigle et al., 1990).

Effect of AM fungi and citrus rootstocks on the growth of Thai honey tangerine scions

Seven month old plants of five citrus species with and without AM fungal inoculation used as rootstocks were Cleopatra tangerine, lime, pomelo, Swingle citrumelo and Troyer citrange in four replications. The plants were grown in natural conditions in a mesh enclosure. Scions of Thai honey tangerine were grafted on the citrus rootstocks. Three months after grafting dry weight of the scions were determined. After drying, the scions were ground and the nutrient contents were analyzed for N by Kjeldahl method (Jackson, 1967), P and K by Dry ashing and molypdovanado-phosphoric acid method, Ca and Mg by Flame-atomic absorption spectrophotometer method (Delhaize et al., 1984).

Statistical analysis

Statistical tests were performed with SPSS software version 10.0. Analysis of variance (ANOVA) of the data was used to test the effects of the factors. Duncan's Multiple Range Test ($p \leq 0.05$) was used to compare the means of the treatments.

RESULTS AND DISCUSSION

Effects of AM fungi on the resistance to *P. parasitica* in different citrus seedlings

Plant species and varieties may differ in their resistance to pathogens. This experiment showed that in the absence of AM fungi, Thai honey tangerine was most susceptible to the root rot pathogen, *P. parasitica* whereas, Cleopatra tangerine was the most resistant to *P. parasitica* (Table 1). Colburn and Graham (2007) reported that Cleopatra tangerine rootstock was more resistant to *P. parasitica* than Troyer citrange rootstock. It is known that plants have their own immune system including physical and chemical barriers and several active mechanisms (Kachroo and Kachroo, 2009). However, this experiment showed that AM fungi significantly reduced disease caused by *P. parasitica* in all the citrus plants. Induced systemic resistance can be activated upon colonization of roots by nonpathogenic microbes (Van Loon et al., 1998).

Disease rating showed that the severity level was significantly depressed by AM fungi. Colonization by AM fungi in the citrus roots varied from 47.0 to 88.8%. The percentage of AM colonization in the citrus roots with

Table 1. Effect of AM fungi on disease index of different citrus seedlings inoculated with *P. parasitica*.

Citrus seedling	Disease index*	
	P. parasitica	**AM fungi and *P. parasitica***
Thai honey tangerine	2.7^{a}	0.4^{d}
Cleopatra tangerine	1.1^{c}	0.1^{d}
Troyer citrange	2.5^{a}	0.7^{cd}
Swingle citrumelo	1.6^{b}	0.9^{c}
Lime	2.5^{a}	0.2^{d}
Pomelo	1.6^{b}	0.3^{d}

Means followed by different letters are significantly different (p<0.05) by Duncan's Multiple Range Test. *Disease index was based on levels of visible symptoms of the disease that showed dulling, yellowing and browning of leaves with some eventually dropping off. The disease was rated on a scale of 0 to 5, with 0 = no visible symptom; 1 = symptom on about 20% of the leaves, through to 5 = symptom on all leaves.

Table 2. Root colonization by AM fungi in different citrus seedlings with and without *P. parasitica*.

Citrus seedling	Root colonization of AM fungi (%)	
	AM fungi	**AM fungi and *P. parasitica***
Thai honey tangerine	69.1^{b} ± 7.4	71.4^{b} ± 7.1
Cleopatra tangerine	47.0^{c} ± 3.6	49.0^{c} ± 4.8
Troyer citrange	64.5^{b} ± 5.3	72.5^{b} ± 2.0
Swingle citrumelo	74.8^{ab} ± 4.6	83.7^{a} ± 6.7
Lime	86.7^{a} ± 6.9	88.8^{a} ± 2.9
Pomelo	72.1^{b} ± 3.6	71.0^{b} ± 2.8

Values are means ± standard errors. Means with different letters are significantly different (p<0.05) by Duncan's multiple range test.

P. parasitica was similar to the citrus roots without *P. parasitica*. Lime had the highest AM colonized roots whereas root colonization of AM fungi was lowest in Cleopatra tangerine (Table 2). Interactions between plants and microbes may be pathogenic or symbiotic. Detection of both pathogenic and symbiotic microbes suggests the activation of a similar set of genes (Zhao and Qi, 2008). Root colonization of AM fungi could reduce disease severity of pathogens via several mechanisms including induced disease resistance, increasing the nutrient uptake and increasing plant growth (Sundaresan et al., 1993; Trotta et al., 1996; Ozgonen and Erkilic, 2007; Kapoor, 2008).

The disease was closely correlated (r = 0.887, p<0.001) with log number of zoospores of *P. parasitica*. Zoospores of *P. parasitica* in the soil inoculated with AM fungi were fewer than those without AM fungi by several orders of magnitude (Figure 1). Reduction of zoospores of *P. parasitica* in the pots may be due to the competition for substrates with AM fungi or resistance of the citrus plants after inoculation with AM fungi. Norman and Hooker (2000) found that in the exudates from strawberry roots colonized by *G. etunicatum* and *G. monosporum*, sporulation of *Phytophthora fragariae* was reduced relative to sporulation in the presence of uncolonized roots by the AM fungi.

Effects of AM fungi and *P. parasitica* on the growth of citrus seedlings

Plant growth responses to AM fungi were different among genotypes. Lime responded the best to AM fungi followed by Troyer citrange, Swingle citrumelo and pomelo but Thai honey tangerine and Cleopatra tangerine did not respond for growth to AM fungi (Table 3). Although, AM fungi are mutualistic symbiosis of most terrestrial plants (Smith and Read, 1997), different plant species have different responses to AM fungi (Requena et al., 2001; Jifon et al., 2002). Whereas, inoculation with AM fungi and *P. parasitica* significantly increased shoot dry weight of lime seedling and slightly increased shoot dry weight of Troyer citrange and pomelo but did not have significant effect on shoot dry weight of the other citrus seedlings. However, shoot dry weight of lime inoculated with AM fungi and *P. parasitica* was lower than the one inoculated with only AM fungi.

In this experiment, there were some variation mechanisms depending on the genotypes of the citrus seedlings. Dry weight of Thai honey tangerine inoculated

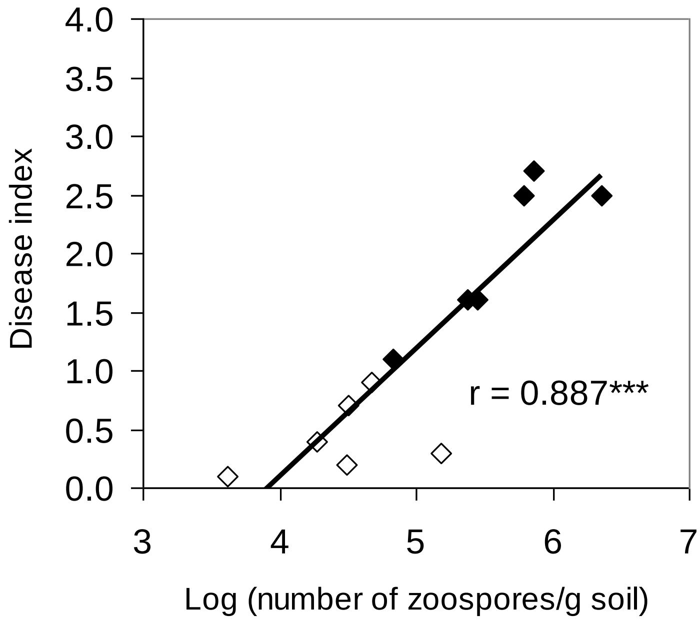

Figure 1. Correlation between disease level of the citrus seedlingss and the amount of zoospores of *P. parasitica*, with (◇) and without (◆) AM fungi. (***, correlation significant at p<0.001).

Table 3. Effect of AM fungi and *P. parasitica* on shoot dry weight of citrus seedlings.

Citrus seedling	Shoot dry weight of citrus seedlings (g)			
	Control	**AM fungi**	***P. parasitica***	**AM fungi and *P. parasitica***
Thai honey tangerine	$0.6^{d} \pm 0.1$	$0.6^{d} \pm 0.3$	$0.6^{d} \pm 0.4$	$1.4^{cd} \pm 1.1$
Cleopatra tangerine	$0.7^{d} \pm 0.2$	$1.4^{cd} \pm 0.4$	$1.1^{cd} \pm 1.0$	$1.9^{cd} \pm 1.0$
Troyer citrange	$0.8^{d} \pm 0.5$	$5.0^{ab} \pm 2.0$	$0.8^{d} \pm 0.7$	$2.8^{bcd} \pm 1.4$
Swingle citrumelo	$1.6^{cd} \pm 1.0$	$4.3^{b} \pm 2.3$	$1.2^{cd} \pm 0.7$	$1.4^{cd} \pm 0.6$
Lime	$1.8^{cd} \pm 1.1$	$7.0^{a} \pm 3.4$	$1.5^{cd} \pm 0.8$	$4.8^{b} \pm 3.5$
Pomelo	$0.6^{d} \pm 0.5$	$3.3^{bc} \pm 2.1$	$1.0^{d} \pm 0.3$	$2.3^{bcd} \pm 0.3$

Values are means ± standard errors. Means with different letters are significantly different (p<0.05) by Duncan's multiple range test.

with AM fungi and *P. parasitica* was higher than the one inoculated only AM fungi but it was with high standard error of the mean. Inoculation with *P. parasitica* did not have significant effect on shoot dry weight of non-mycorrhizal citrus seedlings although there was some variation in shoot dry weight of Cleopatra tangerine which was slightly higher than the one without *P. parasitica*.

Root dry weight of non-mycorrhizal seedlings with and without *P. parasitic* also did not differ significantly. This may be because pathogenicity of *P. parasitica* still had no effect on dry weight of the citrus seedlings after inoculation within one month. AM fungi increased root dry weight of lime, Troyer citrange, Swingle citrumelo and pomelo (Table 4). The higher amount of roots increase nutrient uptake for the growth of shoots. Furthermore, the hyphae of AM fungi are both in the internal and external roots of the host plants. External hyphae of AM fungi increase mineral uptake for growth of the host plants (Frey and Schuepp, 1993).

Effects of AM fungi and citrus rootstocks on the growth and nutrient contents of Thai honey tangerine scions

Beneficial effects of AM fungi on the rootstocks were detected on Thai honey tangerine scions at three months after grafting on seven-month old seedlings of Cleopatra tangerine, lime, pomelo, Swingle citrumelo and Troyer citrange rootstocks. The scion of Thai honey tangerine on the different citrus rootstocks differed markedly in their growth response to AM fungi (Table 5). Without AM fungi,

Table 4. Effect of AM fungi and *P. parasitica* on root dry weight of citrus seedlings.

Citrus seedling	Root dry weight of citrus seedlings (g)			
	Control	AM fungi	*P. parasitica*	AM fungi and *P. parasitica*
Thai honey tangerine	$0.4^{d} \pm 0.2$	$0.8^{d} \pm 0.3$	$0.5^{d} \pm 0.2$	$1.6^{cd} \pm 1.2$
Cleopatra tangerine	$1.1^{d} \pm 0.3$	$1.1^{d} \pm 0.5$	$1.1^{d} \pm 0.8$	$1.3^{cd} \pm 0.9$
Troyer citrange	$1.4^{cd} \pm 0.3$	$3.4^{ab} \pm 1.2$	$1.0^{d} \pm 0.7$	$1.2^{cd} \pm 0.8$
Swingle citrumelo	$1.4^{cd} \pm 0.5$	$3.1^{abc} \pm 1.5$	$1.2^{cd} \pm 0.7$	$1.1^{d} \pm 0.4$
Lime	$2.3^{bcd} \pm 0.8$	$5.0^{a} \pm 2.5$	$2.0^{bcd} \pm 0.2$	$3.5^{ab} \pm 1.3$
Pomelo	$1.2^{cd} \pm 0.5$	$2.3^{bcd} \pm 0.8$	$1.7^{bcd} \pm 0.9$	$1.5^{cd} \pm 0.3$

Values are means ± standard errors. Means with different letters are significantly different (p<0.05) by Duncan's multiple range test.

Table 5. Effect of AM fungi and citrus rootstocks on the growth of Thai honey tangerine scions.

Citrus root stock	Root dry weight of rootstock (g)	Growth of Thai honey tangerine scion	
		Height of scion (cm)	Dry weight of scion (g/plant)
Cleopatra tangerine	$44.7^{c} \pm 1.7$	$15.5^{bc} \pm 0.8$	$20.7^{c} \pm 0.5$
Cleopatra tangerine + AM	$44.4^{c} \pm 0.4$	$18.2^{bc} \pm 3.5$	$20.7^{c} \pm 0.7$
Troyer citrange	$44.8^{c} \pm 0.3$	$18.5^{bc} \pm 3.3$	$21.0^{c} \pm 0.5$
Troyer citrange +AM	$50.5^{ab} \pm 5.7$	$24.0^{ab} \pm 5.9$	$23.7^{b} \pm 2.1$
Swingle citrumelo	$43.8^{c} \pm 2.2$	$9.3^{c} \pm 0.6$	$16.8^{d} \pm 3.5$
Swingle citrumelo+AM	$47.1^{bc} \pm 2.8$	$34.6^{a} \pm 8.4$	$24.3^{b} \pm 3.9$
Lime	$52.0^{ab} \pm 5.0$	$25.6^{ab} \pm 9.0$	$23.0^{b} \pm 6.4$
Lime + AM	$54.8^{a} \pm 4.1$	$36.5^{a} \pm 7.7$	$26.9^{a} \pm 1.7$
Pomelo	$49.2^{bc} \pm 5.6$	$17.0^{bc} \pm 7.2$	$14.0^{e} \pm 5.4$
Pomelo + AM	$50.8^{ab} \pm 4.2$	$19.0^{bc} \pm 5.3$	$21.0^{c} \pm 3.5$

Values are means ± standard errors. Means in the same column followed by different letters are significantly different (p< 0.05) by Duncan's multiple range test.

the scion was the shortest on the Swingle citrumelo rootstock and the tallest on the lime rootstock. However, AM fungi significantly increased the height of the scion on Swingle citrumelo rootstock by nearly four folds of the non-mycorrhizal one, while the effect on the height of the scion on the other rootstocks was much less.

The benefit from AM fungi was also obvious in the increase of the shoot dry weight of the scion at different rates on lime, Swingle citrumelo, Troyer citrange and pomelo rootstocks but not on Cleopatra tangerine. The Thai honey tangerine scion grew best on the lime root stock with AM fungi. Interactions between rootstocks and scions occur in many relations such as compatibility of rootstocks and scions, efficiency of water and nutrient uptake of rootstocks that affect the growth of the scions (Taylor and Dimsey, 1993; Almansa, 2002).

Inoculation with AM fungi differently increased N, P, K, Ca and Mg contents of the Thai honey tangerine scions on different rootstocks (Table 6). The nutrient contents except K of the scion on the Troyer citrange rootstock inoculated with AM fungi were significantly higher than the non-mycorrhizal one. Whereas, the nutrient contents except N of the scion on lime rootstock inoculated with AM fungi were significantly higher than the non-mycorrhizal one, especially P content of the scion on mycorrhizal lime rootstock was about 3.8 folds of the non-mycorrhizal one. The Thai honey tangerine scions had the highest P and K contents on the lime rootstock inoculated with AM fungi. There are many reports that AM fungi increased many kinds of nutrient contents according to the host plants and soil conditions (Marschner and Dell, 1994; Clark and Zeto, 1996; Youpensuk et al., 2006).

This experiment showed that AM fungi significantly increased the N, P, K, Ca and Mg contents in the Thai honey tangerine scions on the citrus rootstocks but AM fungi had no effect on the nutrient contents of the tangerine scion on Cleopatra tangerine rootstock. However, non-mycorrhizal Cleopatra tangerine rootstock was more efficient in Ca and Mg accumulation in the Thai honey tangerine scions than the other non-mycorrhizal citrus rootstocks. Pectic substances that cross-link with calcium in the middle lamella become calcium pectate, which increase the strength of plant cell resistance to plant pathogens. Moreover, cytosolic Ca^{2+} in plant is a component of signals in resistance to plant pathogens

Table 6. Effect of AM fungi and citrus rootstocks on the nutrient contents of Thai honey tangerine scions.

Citrus root stock	Nutrient contents of Thai honey tangerine scions (mg/plant)				
	N	P	K	Ca	Mg
Cleopatra tangerine	494.9[de]	37.5[cd]	358.2[de]	228.6[a]	47.9[abc]
Cleopatra tangerine + AM	500.1[cde]	31.1[e]	347.8[de]	239.2[a]	51.9[ab]
Troyer citrange	502.6[cde]	33.0[e]	563.3[b]	176.0[b]	30.9[e]
Troyer citrange + AM	580.2[ab]	39.6[c]	567.3[b]	239.3[a]	59.5[a]
Swingle citrumelo	439.5[e]	25.1[f]	388.6[d]	149.3[c]	29.8[e]
Swingle citrumelo + AM	623.3[a]	44.7[b]	542.7[bc]	251.1[a]	44.8[bcd]
Lime	533.4[bcd]	29.9[ef]	561.8[b]	158.7[bc]	33.1[de]
Lime + AM	569.4[abc]	113.0[a]	767.2[a]	241.6[a]	46.9[abc]
Pomelo	312.9[f]	15.2[g]	315.7[e]	106.0[d]	25.5[e]
Pomelo + AM	511.1[bcd]	37.2[cd]	488.7[c]	160.1[bc]	37.7[cde]

Means in the same column followed by different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

(Scheel, 1998; Sanders et al., 2002). Therefore, the increase of Ca contents in plants may increase the disease resistance in Cleopatra tangerine.

Conclusion

This research has shown that different citrus genotypes responded differently to AM fungi and *P. parasitica*. In the absence of AM fungi, Thai honey tangerine was most susceptible and Cleopatra tangerine was most resistant to *P. parasitica*. Colonization by AM fungi significantly lowered the number of zoospores of the root rot pathogen and disease symptoms on all the citrus plants. AM fungi enhanced the greatest growth in lime seedlings followed by Troyer citrange, Swingle citrumelo and pomelo but Thai honey tangerine and Cleopatra tangerine seedlings did not respond for growth to AM fungi. AM fungi significantly increased most of the N, P, K, Ca and Mg contents of the Thai honey tangerine scions on the citrus rootstocks except on Cleopatra tangerine rootstock. The scion of Thai honey tangerine grew best on the lime root stock inoculated with AM fungi.

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