



Genotypic Variation in Responses of *Citrus* spp. to Arbuscular Mycorrhizal Fungi

Somchit Youpensuk (Corresponding author)

Department of Biology, Faculty of Science, Chiang Mai University
Chiang Mai 50200, Thailand

Tel: 66-053-941-946-8 ext. 143 E-mail: scboi027@chiangmai.ac.th

Sittichai Lordkaew

Multiple Cropping Centre, Faculty of Agriculture, Chiang Mai University,
Chiang Mai 50200, Thailand

Tel: 66-081-796-0449 E-mail: s.lord@chiangmai.ac.th

Benjavan Rerkasem

Department of Agronomy, Faculty of Agriculture, Chiang Mai University,
Chiang Mai 50200, Thailand

Tel: 66-086-182-4678 E-mail: benjavan@chiangmai.ac.th

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Abstract

Thailand is part of Southeast Asia that covers the center of diversity of citrus species, where various species of the genus are widely grown. One of the most common is tangerine (*Citrus reticulata*), which is commonly grown by grafting on rootstocks of different tangerine varieties or other citrus species. The objective of this study is to investigate responses of some *Citrus* spp. seedlings to arbuscular mycorrhizal (AM) fungi, and thus their potential as rootstocks. The experiment was done with four tangerine varieties, Cleopatra, Fremont, Ocean and Sainamphung; and four other citrus species, lime (*C. aurantifolia*), pomelo (*C. maxima*), sweet orange (*C. sinensis*) and Troyer citrange (*Citrus sinensis*×*Poncirus trifoliata*), in pots for five months. Roots of non-inoculated plants were not infected with AM fungi, while inoculated plants were heavily infected with AM fungi, and contained 14-28 AM spores per 10 g of rhizosphere soil. Most of the citrus responded positively to AM fungi, but with different magnitudes among the varieties and species. Lime and pomelo seedlings were fast growing compared to other citrus species. Total dry weight and N, P, K and Mg contents were increased most strongly by AM fungi in lime, pomelo and tangerine varieties Ocean, Fremont and Sainamphung, but little or none in Cleopatra, Troyer and sweet orange. Lime was the most outstanding in the response to AM fungi, followed by Ocean tangerine and pomelo. The potential of lime, pomelo and Ocean tangerine as rootstock for tangerine should be further investigated.

Keywords: Arbuscular mycorrhizal fungi, *Citrus* spp., Response

1. Introduction

Southeast Asia is generally considered the center of diversity of citrus (Moore, 2001). Various kinds of citrus plants are grown in all regions of Thailand, one of the most widely grown is tangerine (*C. reticulata*) especially the variety Sainamphung. Tangerine is commonly grown by grafting on rootstocks of different tangerine varieties or other citrus species. Tangerine variety Cleopatra and a hybrid citrange or Troyer (*Citrus sinensis*×*Poncirus trifoliata*) are used widely as rootstock in Sainamphung tangerine orchards in Thailand. Arbuscular mycorrhizal (AM) fungi are mutualistic associations with plant roots. They improve the nutritional status of plants resulting in increased growth of the host plants, and they can also improve soil structure (Douds and Millner, 1999). AM fungi are an important part of sustainable agricultural systems. Youpensuk et al. (2008) reported that twenty-two species of AM fungi were found in tangerine

orchards of Chiang Mai province and they increased growth of air layered tangerine variety Sainamphung especially in pots applied only N without P fertilizer. The objective of this study is to investigate responses to AM fungi of seedlings of tangerine varieties and other common citrus species, and thus their potential as rootstocks.

2. Materials and Methods

2.1 Preparing citrus seedlings

Tangerine (*C. reticulata*) varieties examined in this experiment were Cleopatra, Fremont, Ocean and Sainamphung, and the other common citrus plants were lime (*C. aurantifolia*), pomelo (*C. maxima*), sweet orange (*C. sinensis*) and a hybrid citrange or Troyer (*Citrus sinensis*×*Poncirus trifoliata*). The plants were grown from seeds that had outer seed coats were peeled off before germination in sterile soil in plastic trays and watered twice a day.

2.2 The responses of *Citrus* spp. seedlings to AM fungi

One month old seedlings were transplanted into drainable plastic pots containing 6 kg sterile soil, with one seedling per pot. The soil was a sandy clay loam with pH of 6.0. The soil contained 0.90 g/kg total N, 4.1 mg/kg available P, 53.0 mg/kg extractable K, and 18.5 g/kg organic matter. Spores of mixed AM fungal species were collected from the rhizosphere of *Citrus* spp. in northern Thailand. For inoculated treatments, three hundred spores of mixed species of AM fungi were inoculated to the planting hole in each pot. All treatments had four replications. Seedlings were watered once a day. Five months after transplanting, shoot height was measured for each plant. Shoots were separated from roots at the soil surface and dried at 70°C for three days to evaluate for shoot dry weight. Soil in each pot was divided into two subsamples. Roots were washed from each one soil subsample and dried at 70°C for three days to evaluate for root dry weight. After drying, shoot and root samples were ground and analyzed for N contents in citrus plants by Kjeldahl method. Dry ashes of the samples of citrus plants were evaluated for P by molybdovanado-phosphoric acid method, and evaluated for K and Mg by atomic absorption spectrophotometer.

2.3 Assessment of root colonization and spore density of AM fungi

Soil and root samples from the second subsamples of each pot were used for assessment of root colonization and spore density of AM fungi. Fifty g of soil sample from each pot was used to assess spore density and identification of the AM fungi. The soil samples were wet sieved through 750, 250, 100, and 53 µm mesh sieves. The sieved soil on each 250, 100, and 53 µm mesh was centrifuged at 2000 rpm for 5 min and floating particles removed. The soil was suspended in 50% sucrose and centrifuged one min at 2000 rpm. After centrifugation, spores in the supernatant were poured over the finest 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 and counted under a stereomicroscope. The spores of AM fungi were identified according to morphological characteristics of AM fungal descriptions (Schenck and Perez, 1988; INVAM website, 2008).

Root samples were washed with tap water and cut into about 1 cm in length, cleared in 10% KOH at 121°C for 15 min, washed over a sieve with tap water, and stained with 0.05% trypan blue in lactoglycerol at 121°C for 15 min. The stained root segments were randomly picked with fine tip forceps and mounted on slides. Thirty pieces of root segments from each sample were assessed root colonization of AM fungi according to the method of McGonigle et al (1990) under compound microscope.

2.4 Statistical analysis

The data were analyzed with SPSS software program for analysis of variance (ANOVA). Duncan's Multiple Range Test at $P \leq 0.05$ was used to determine significances of treatment means.

3. Results and Discussion

Roots of non-inoculated plants were not infected with AM fungi. Percentage of root colonization of AM fungi in roots of inoculated citrus plants was very high from 75 – 96% (Table 1). Spore densities in the pots were about 14-28 spores per 10 g soil. Spores production may not correlate to percentage of root colonization it depends on AM fungal ability to produce spores in each soil condition (Smith and Read, 1997; Youpensuk et al., 2006). Twenty species of AM fungi were found in pots of inoculated citrus plants after five months of inoculation (Table 2). They were in three genera of *Acaulospora* (7 species), *Glomus* (12 species) and *Scutellospora* (1 species). The most AM species frequently found in all pots of inoculated citrus plants were *Acaulospora scrobiculata*, *Glomus etunicatum* and *G. mosseae*. This was similar to the report of Youpensuk et al. (2008) which found that *G. etunicatum* and *A. scrobiculata* were the most frequently found in tangerine orchards in Chiang Mai province. In this experiment, some 13-14 species of AM fungi were found in the rhizosphere of tangerine. There were some difference in the AM fungal species among the different varieties of tangerine, such as *G. aggregatum* was found only in the tangerine variety Cleopatra but *A. rugosa* was not found in this variety while it was found in the other varieties, and *A. delicata* was found in only tangerine variety Ocean. There were 2-3 more AM fungal species in the rhizosphere of the other citrus species. The most abundant in AM fungal species was in the hybrid citrange or Troyer (Table 2). Bever (2002) reported that although AM fungal species can associate with all host but they have host-specific differences in their sporulation growth rates. Although they were not very different in

number of AM fungal species and percentage of root colonization but significant variation in responses to AM fungi were found between the tangerine varieties and among the citrus species (Table 3). Inoculation by AM fungi generally increased plant height in the citrus, but most strongly in pomelo, lime and Ocean tangerine. Inoculation with AM fungi increased shoot dry weight most strongly, by doubling the shoot dry weight or more, in two tangerine varieties, Fremont and Ocean, and also in lime and pomelo, less effect on the tangerine varieties Cleopatra and Sainamphung, but had little effect on sweet orange and Troyer. Root dry weight of AM inoculated lime was three times that in non-inoculated. AM inoculation did not have significant effect on root dry weight of any of the tangerine and other citrus species. Root to shoot ratios of inoculated treatments of all tangerine varieties and pomelo tended to be lower than those of non-inoculated treatments. This may be the result of greater nutrient uptake efficiency by the external hyphae of mycorrhizal roots, which resulted in increased shoot growth of the host plant. Mycorrhizal plants are frequently found to have lower root to shoot ratios than non-mycorrhizal plants (Marschner et al., 1996; Youpensuk et al., 2005). Lime was an exception, as both its root growth was tripled by AM inoculation while the shoot growth was only doubled, which resulted in the root to shoot ratio being increased by AM inoculation.

Comparative responses to AM fungi in these citrus species and tangerine varieties can be seen more clearly by comparing plant total dry weight with and without mycorrhiza (Table 4). Lime responded most strongly to AM fungi with a 115.5% increase in total dry weight, followed closely by tangerine Ocean with 97.7% and pomelo with 91.4% increase in dry weight. Significant, although lower, responses to AM fungi were found in tangerine Fremont (78.4%) and Sainamphung (64.9%). The response to AM fungi was not significant in Cleopatra tangerine, sweet orange and Troyer. The low response to AM fungi in Troyer has been previously reported by Camprubi and Calvet (1996). These authors, however, also reported that rootstocks of sour orange and Cleopatra were more mycorrhizal dependent than Troyer and Swingle citrumelo (*C. paradise* × *P. trifoliata*). The lack of response to AM fungi in sweet orange agrees with the report of Jifon et al. (2002), who reported that sweet orange did not respond to *Glomus intraradices* while sour orange or *C. aurantium* had about 15% of mycorrhizal dependence in high level of P and 70 Pa of CO₂. The results indicated that varieties or species of plants can respond differently to AM fungi. Soil condition also affect to responses of plants to AM fungi such as levels of available P in soil that AM plants more response to AM fungi in low P soil than in high P soil (Graham et al., 1997). In addition to variation in the effect of AM fungi on plant dry weight among the citrus species and tangerine varieties, AM fungi also had different effects on nutrients uptake of the different citrus plants that were sometimes similar to and sometimes different from the effects on dry weight (Table 5). Although AM fungi had little effect on dry weight of Cleopatra tangerine, but it significantly increased N, P and K content of the host plant. Similarly AM fungi significantly increased P content of sweet orange and N and P content of Troyer even though it did not affect their dry weight. The effect of AM fungi on nutrient uptake in some of the more responsive varieties of tangerine and species of citrus was very large. For example, P content of Fremont tangerine, lime and pomelo was increased by more than 300% by AM fungi. The largest increases in the uptake of these nutrients by AM fungi were found in Fremont tangerine, lime and pomelo, while the effect on Troyer was small and on Sweet orange nil. Troyer and Cleopatra are tolerant to *Phytophthora* spp. that cause root rot of plants (Graham and Timmer, 2009). Therefore, they are widely used in Thailand as rootstock for Sainamphung, but their limited response to AM fungi is a cause for concern. Should this response continue into trees in the orchards, it would mean that benefits from AM fungi will be minimal in commercial tangerine orchards. Many experiments reported that AM fungi increased P contents of the host plants. But AM fungi may or may not increase uptake of these N, K and Mg, depending on host plants, species of AM fungi and soil conditions (Marschner and Dell, 1994; Taylor and Harrier, 2001; Rutto et al., 2002). Wu and Xia (2006) reported that level of Mg in leaves of tangerine inoculated with AM fungi was higher in AM seedlings than those in non-AM seedlings under well-watered and water stress conditions. While the levels of K and Ca in leaves and roots were significantly higher in AM seedlings than those in non-AM seedlings only under well-water conditions.

4. Conclusions

All of the tangerine varieties and other citrus species studied were well colonized by AM fungi. Judging by spore morphology a whole range of AM fungi was found in the rhizosphere of these citrus. All of citrus also benefited from association with the AM fungi, but with different magnitudes in different varieties and species. Lime was the most outstanding in the response to AM fungi, followed by Ocean tangerine and pomelo. The limited response to AM fungi in Troyer and Cleopatra tangerine that are used as rootstocks in commercial tangerine orchards suggests a re-evaluation of tangerine rootstocks may be worthwhile. On the other hand the potential of lime, pomelo and Ocean tangerine as rootstock for tangerine should be further investigated.

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Table 1. Means of root colonization and spore densities of arbuscular mycorrhizal fungi in pots of inoculated citrus plants

Species or variety of citrus plant	Mean of root colonization (%)	Spore density (spores/10 g soil)
Cleopatra	75.0b	28a
Fremont	95.5a	14d
Ocean	90.6a	16bcd
Sainamphung	90.6a	23ab
Lime	91.8a	21abc
Pomelo	96.0a	22abc
Sweet Orange	95.1a	21abc
Troyer	94.5a	15cd

Means in the same column followed by different letters are significantly different ($P \leq 0.05$).

Table 2. Species of arbuscular mycorrhizal fungi found in pots of inoculated citrus plants, five months after inoculation with AM fungi

Genera of AM fungi		Species or variety of citrus plants										
	Cleopatra	Fremont	Ocean	Sainumphung	Lime	Pomelo	Sweet Orange	Troyer				
<i>Acaulospora</i>	-	-	<i>A. delicata</i>	-	<i>A. delicata</i>	<i>A. delicata</i>	<i>A. delicata</i>	-				
	<i>A. denticulata</i>	<i>A. denticulata</i>	<i>A. denticulata</i>	<i>A. denticulata</i>	<i>A. denticulata</i>	<i>A. denticulata</i>	<i>A. denticulata</i>	<i>A. denticulata</i>				
	<i>A. lacunosa</i>	<i>A. lacunosa</i>	<i>A. lacunosa</i>	<i>A. lacunosa</i>	<i>A. lacunosa</i>	<i>A. lacunosa</i>	<i>A. lacunosa</i>	<i>A. lacunosa</i>				
	<i>A. longula</i>	<i>A. longula</i>	<i>A. longula</i>	<i>A. longula</i>	<i>A. longula</i>	<i>A. longula</i>	<i>A. longula</i>	<i>A. longula</i>				
	<i>A. morrowiae</i>	<i>A. morrowiae</i>	<i>A. morrowiae</i>	<i>A. morrowiae</i>	<i>A. morrowiae</i>	<i>A. morrowiae</i>	<i>A. morrowiae</i>	<i>A. morrowiae</i>				
	-	<i>A. rugosa</i>	<i>A. rugosa</i>	<i>A. rugosa</i>	<i>A. rugosa</i>	<i>A. rugosa</i>	<i>A. rugosa</i>	<i>A. rugosa</i>	<i>A. rugosa</i>			
	<i>A. scrobiculata</i>	<i>A. scrobiculata</i>	<i>A. scrobiculata</i>	<i>A. scrobiculata</i>	<i>A. scrobiculata</i>	<i>A. scrobiculata</i>	<i>A. scrobiculata</i>	<i>A. scrobiculata</i>	<i>A. scrobiculata</i>			
	<i>G. aggregatum</i>	-	-	-	-	<i>G. aggregatum</i>	<i>G. aggregatum</i>	<i>G. aggregatum</i>	<i>G. aggregatum</i>			
	-	-	-	-	<i>G. australe</i>	-	-	-	-			
	<i>G. caledonium</i>	<i>G. caledonium</i>	<i>G. caledonium</i>	<i>G. caledonium</i>	<i>G. caledonium</i>	<i>G. caledonium</i>	<i>G. caledonium</i>	<i>G. caledonium</i>	<i>G. caledonium</i>			
<i>G. claroideum</i>	<i>G. claroideum</i>	<i>G. claroideum</i>	<i>G. claroideum</i>	<i>G. claroideum</i>	<i>G. claroideum</i>	<i>G. claroideum</i>	<i>G. claroideum</i>	<i>G. claroideum</i>				
-	-	-	-	-	-	-	-	<i>G. clavisorum</i>				
-	-	-	-	-	-	-	-	<i>G. constrictum</i>				
<i>G. etunicatum</i>	<i>G. etunicatum</i>	<i>G. etunicatum</i>	<i>G. etunicatum</i>	<i>G. etunicatum</i>	<i>G. etunicatum</i>	<i>G. etunicatum</i>	<i>G. etunicatum</i>	<i>G. etunicatum</i>				
<i>G. fasciculatum</i>	<i>G. fasciculatum</i>	<i>G. fasciculatum</i>	<i>G. fasciculatum</i>	<i>G. fasciculatum</i>	<i>G. fasciculatum</i>	<i>G. fasciculatum</i>	<i>G. fasciculatum</i>	<i>G. fasciculatum</i>				
<i>G. invernatum</i>	<i>G. invernatum</i>	<i>G. invernatum</i>	<i>G. invernatum</i>	<i>G. invernatum</i>	<i>G. invernatum</i>	<i>G. invernatum</i>	<i>G. invernatum</i>	<i>G. invernatum</i>				
<i>G. macrocarpum</i>	<i>G. macrocarpum</i>	<i>G. macrocarpum</i>	<i>G. macrocarpum</i>	<i>G. macrocarpum</i>	<i>G. macrocarpum</i>	<i>G. macrocarpum</i>	<i>G. macrocarpum</i>	<i>G. macrocarpum</i>				
<i>G. mosseae</i>	<i>G. mosseae</i>	<i>G. mosseae</i>	<i>G. mosseae</i>	<i>G. mosseae</i>	<i>G. mosseae</i>	<i>G. mosseae</i>	<i>G. mosseae</i>	<i>G. mosseae</i>				
-	-	-	-	-	-	-	-	<i>G. multicaule</i>				
								<i>S. heterogama</i>				
Total of AM species	13	13	14	13	15	15	16	17				

Table 3. Effect of AM fungi on height, shoot and root dry weight (DW) of tangerine varieties and other citrus plants, five months after inoculation

Species or variety of citrus plant	Height (cm)	Shoot DW (g/plant)	Root DW (g/plant)	Root:shoot
Cleopatra (M-)	23.67f	1.79e	1.47d	0.82
Cleopatra (M+)	32.73de	2.73de	1.32d	0.48
Fremont (M-)	29.23ef	2.34de	2.06cd	0.88
Fremont (M+)	37.78cd	4.76bc	3.09cd	0.65
Ocean (M-)	29.98e	2.63de	1.69cd	0.64
Ocean (M+)	46.60bc	6.14bc	2.38cd	0.39
Sainamphung (M-)	32.90de	1.84e	1.98cd	1.08
Sainamphung (M+)	33.10de	3.35de	2.95cd	0.88
Lime (M-)	48.80bc	5.02bc	2.45b	0.49
Lime (M+)	65.93a	11.79a	7.31bc	0.62
Pomelo (M-)	39.35bc	4.97b	3.87ab	0.78
Pomelo (M+)	62.55a	11.08a	5.84a	0.53
Sweet orange (M-)	33.85cd	5.44b	3.61ab	0.66
Sweet orange (M+)	44.38bc	5.53b	3.68ab	0.67
Troyer (M-)	51.00bc	3.80cd	2.40cd	0.63
Troyer (M+)	55.13b	3.87cd	2.40cd	0.62
Analysis of variance				
Citrus	***	***	***	
Inoculation	***	***	NS	
Citrus × Inoculation	NS	***	NS	

M-, non-inoculated with AM fungi; M+, inoculated with AM fungi. Means in the same column followed by different letters are significantly different ($P \leq 0.05$). ***, significant at $P \leq 0.001$; NS, not significant.

Table 4. Total dry weight and response to mycorrhizal fungi of tangerine varieties and other citrus plants, five months after inoculation with arbuscular mycorrhizal fungi

Plant	Total dry weight (g/plant)		Response to mycorrhizal* (%)
	Non-mycorrhizal plant	Mycorrhizal plant	
Tangerine			
Cleopatra	3.26cA	4.04cA	23.9
Fremont	4.40cB	7.85bcA	78.4
Ocean	4.31cB	8.52bcA	97.7
Sainamphung	3.82cB	6.30cA	64.9
Other citrus spp.			
Lime	7.47abB	16.10aA	115.5
Pomelo	8.84aB	16.92aA	91.4
Sweet Orange	9.05aA	9.16bA	1.2
Troyer	6.20bcA	6.27cA	1.1

*Dry weight of mycorrhizal plant as percentage of dry weight of non-mycorrhizal plant. Means of total dry weight followed by different letters (lower case in the same column and capital letter in the same row) were significantly different ($P \leq 0.05$).

Table 5. Effect of arbuscular mycorrhizal fungi on nutrient contents in tangerine varieties and other citrus plants, five months after inoculation.

Species or variety of citrus plant	Nutrient content in citrus plants (mg/plant)			
	N	P	K	Mg
Cleopatra (M-)	47.63b	5.98b	47.25b	8.09a
Cleopatra (M+)	70.85a	12.99a	58.67a	10.93a
Fremont (M-)	59.01b	8.33b	109.84b	9.22b
Fremont (M+)	139.00a	38.19a	153.39a	14.25a
Ocean (M-)	67.41b	8.79b	104.31b	8.56b
Ocean (M+)	134.08a	24.22a	142.00a	13.49a
Sainamphung (M-)	47.51b	10.12b	86.54b	9.72b
Sainamphung (M+)	111.46a	30.11a	124.26a	15.47a
Lime (M-)	104.44b	14.46b	154.56b	14.90b
Lime (M+)	239.31a	62.51a	312.07a	37.32a
Pomelo (M-)	95.91b	10.89b	149.80b	20.00b
Pomelo (M+)	201.97a	44.52a	270.66a	34.02a
Sweet Orange (M-)	141.12a	18.45b	220.37a	17.92a
Sweet Orange (M+)	151.03a	28.73a	161.70b	14.92a
Troyer (M-)	71.72b	15.66b	114.22a	12.56a
Troyer (M+)	107.05a	27.28a	114.16a	12.19a

Means of non-inoculated (M-) and inoculated (M+) with AM fungi in the same tangerine variety or citrus species followed by different letters were significantly different ($P \leq 0.05$).