

## เอกสารอ้างอิง

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## ภาคผนวก

### Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานได้ส่งตีพิมพ์ในวารสารวิชาการนานาชาติ (อยู่ระหว่างการพิจารณาในการตีพิมพ์)
2. การนำผลงานวิจัยไปใช้ประโยชน์
  - ในเชิงวิชาการ โดยใช้ในการพัฒนาการเรียนการสอน และสร้างนักวิจัยใหม่ที่สามารถทำงานวิจัยเกี่ยวกับการนำเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาไปใช้ประโยชน์
  - สามารถนำผลงานวิจัยนี้ไปใช้ประโยชน์ในการปลูกส้มเขียวหวานพันธุ์สายน้ำผึ้ง และลดความรุนแรงของโรคจากการใช้เชื้อราอาร์บัสคูลาร์ไมคอร์ไรซา และการมะนาวแป้นนำมาใช้เป็นต้นตอให้กับส้มเขียวหวานพันธุ์สายน้ำผึ้ง เพื่อเป็นทางเลือกในการลดต้นทุนจากการใช้เมล็ดพันธุ์ผสมลูกผสมเป็นต้นตอ

**Manuscript ที่ใช้ในการส่งตีพิมพ์ในวารสารวิชาการนานาชาติ**

**Effects of arbuscular mycorrhizal fungi on resistance to *Phytophthora parasitica* of *Citrus* spp. and on growth of tangerine variety Sainamphung scions on rootstocks of *Citrus* spp.**

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**Abstract**

Tangerine variety Sainamphung is generally grown by grafting on rootstocks of other variety or other citrus species due to it is susceptible to root rot disease. The objectives of this study are (1) to investigate the effects of arbuscular mycorrhizal (AM) fungi on resistance to *Phytophthora parasitica* and the growth of seedlings of *Citrus* spp. and (2) to study the effect of AM fungi and rootstocks of *Citrus* spp. on growth of the scions of tangerine variety Sainamphung. The citrus species used in this experiment were Common tangerine variety Sainamphung (*Citrus reticulata*) and Cleopatra tangerine (*C. reshni*), lime (*C. aurantifolia*), pomelo (*C. grandis*), Swingle citrumelo (*Citrus paradisi*×*Poncirus trifoliata*) and Troyer citrange (*Citrus sinensis*×*Poncirus trifoliata*).



The results showed that AM fungi reduced disease severity of the citrus plants about 2-16 times of the non-mycorrhizal plants. Citrus plants without AM fungi showed that tangerine variety Sainamphung was the most susceptible to *P. parasitica* followed by Troyer citrange, lime, pomelo and Swingle citrumelo, respectively. Whereas, Cleopatra tangerine was the most resistant to *P. parasitica*. AM fungi improved the highest growth of lime seedling. The effect of AM fungi on the growth of tangerine variety Sainamphung scions which grafting on the citrus rootstocks of Cleopatra tangerine, Troyer citrange, Swingle citrumelo, lime and pomelo was investigated for three months. It was found that the scions of tangerine variety Sainamphung grew best and accumulated the highest phosphorus and potassium contents on the lime rootstock inoculated with AM fungi.

**Keywords:** *Citrus* spp., rootstock, arbuscular mycorrhizal fungi, root rot disease

## Introduction

Tangerine or Common Mandarin (*Citrus reticulata*) has many varieties in the world. Tangerine variety Sainumphung is widely grown in many parts of Thailand. It is a delicious variety but it is susceptible to root rot disease. *Phytophthora parasitica* is the most important soilborne pathogen of the disease. The suitable condition for growth of the fungus is very moist soil. The disease is gradually developed causing brown necrosis on the roots. The visible symptoms of the disease are yellow blight and die of leaves. Therefore, tangerine variety Sainumphung is generally grown by grafting on rootstocks of other citrus species such as Cleopatra Mandarin (*C. reshni*), Swingle citrumelo (*Citrus paradisi*×*Poncirus trifoliata*) and Troyer citrange (*Citrus sinensis*×*Poncirus trifoliata*). Arbuscular mycorrhizal (AM) fungi are mutualistic associations with plant roots. They

enhance mineral uptake through plant roots and reduce disease severity caused by fungal soilborne pathogens (Trotta et al., 1996; Akkopru and Demir, 2006; Ozgonen and Erkilic, 2007). Therefore, AM fungi are an important part of sustainable agricultural systems that have low inputs of chemical fertilizers and pesticides. In this research, we are interested in investigating *Citrus* spp. to be used as rootstocks of tangerine variety Sainamphung, especially for their potential to utilize benefits from association with the AM fungi and resistance to the root rot fungus (*P. parasitica*). The objectives of this research are (1) to investigate the effects of AM fungi on the growth and resistance to *P. parasitica* of seedlings of *Citrus* spp. and (2) to study the effect of AM fungi and *Citrus* spp. as rootstocks on the growth of the scion, tangerine variety Sainamphung.

## Materials and Methods

### Experiment 1. Effects of AM fungi on growth and resistance to *P. parasitica* of citrus seedlings

The citrus species used in this experiment were tangerine variety Sainamphung (*Citrus reticulata*) Cleopatra tangerine (*C. reshni*), lime (*C. aurantifolia*), pomelo (*C. grandis*), Swingle citrumelo (*Citrus paradisi*×*Poncirus trifoliata*) and Troyer citrange (*Citrus sinensis*×*Poncirus trifoliata*). The factorial of this experiment consisted of six citrus species, two treatments of AM inoculation (inoculated and non-inoculated treatments) and two treatments of *P. parasitica* inoculation, (inoculated and non-inoculated treatments) with four replications. Seeds of the *Citrus* spp. used in this experiment were peel off their outer seed coats and the seeds were grown in mixture of sterilized soil and sand (1:1, v/v) in plastic tray. One month old seedlings were

transplanted with one plant per pot which contained with mixture of five kg of mixture sterilized clay loam soil and leaf litter (2:1, v/v). For AM fungal inoculated treatment, about 400 spores of mixed species of AM fungi in soil inoculum were placed under the roots of the seedlings. The plants were watered once a day. Two months after AM fungal inoculation, about  $10^5$  zoospores of *P. parasitica* were inoculated around the root zone of the seedlings.

### **Evaluation the effect of *P. parasitica* on the citrus plants**

Symptom of root rot disease of citrus seedlings was evaluated about one month after *P. parasitica* inoculation. The disease severity based on levels of visible symptom of the disease that showed yellow and brown leaves per entire plant. Soil samples of the *Citrus* spp. were sampling to evaluate zoospores in the pot experiment by using both haemocytometer and spread plate technique for calculation of zoospore numbers.

### **Effect of AM fungi on the citrus plants**

Plant shoot and root dry weight was measured to determine the effect of AM fungi on the growth of the citrus seedlings. In the AM inoculate treatments, roots of the citrus were cleared in 10% KOH, stained with 0.05% trypan blue at 121°C for 15 minutes and determined of AM root colonization as described by Brundrette et al. (1996).

### **Experiment 2. Effect of AM fungi and citrus species as rootstocks on the growth of the scion, tangerine variety Sainamphung.**

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 the same condition and the same time as the above experiment. Scions of tangerine variety Sainamphung from a garden in Chiang Mai province, northern of Thailand were grafted on the citrus rootstocks. Three months after grafting, dry weight, nitrogen, phosphorus, potassium, calcium and magnesium contents of the scions were evaluated.

## Results

### Effects of AM fungi on resistance to *P. parasitica* of citrus seedlings

One month after inoculation with *P. parasitica*, the citrus seedling showed symptoms of some leaves with yellow, dull, brown and fell down. Disease incidence was calculated according from visible symptoms. The results showed that AM fungi reduced disease severity of the citrus plants about 2-16 times of the non-mycorrhizal plants (Table 1). Citrus plants without AM fungi showed that tangerine variety Sainamphung was the most susceptible to *P. parasitica*, it had the highest disease severity followed by Troyer citrange, lime, pomelo and Swingle citrumelo. Whereas, Cleopatra tangerine was the most resistant to *P. parasitica*, especially in Cleopatra tangerine inoculated with AM fungi disease severity was lower than the non-mycorrhizal plant about 16 times.

Zoospores of *P. parasitica* in soil from the pots inoculated with AM fungi were lower than from the pots without AM fungi. The amount of zoospores of *P. parasitica* in the soil had high correlation with disease severity of the citrus plants (Figure 1). Colonization by AM fungi in the citrus roots varied from 47.0% to 88.8% (Table 2). Percentage of AM colonization in the citrus roots with *P. parasitica* was similar to the citrus roots without *P. parasitica*. Lime had the highest AM colonization roots about 88%.



In the other citrus roots had AM colonization about 70% except in Cleopatra tangerine roots that AM colonization was about 48%. However, AM fungi still had effect on reduction of the disease severity as the above mention.

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

In the non-mycorrhizal seedlings with and without *P. parasitica*, shoot dry weight of all citrus species did not have significant difference. Whereas, AM fungi significantly increased shoot dry weight of Troyer citrange, Swingle citrumelo, lime and pomelo, except tangerine variety Sainamphung and Cleopatra tangerine. The citrus plants inoculated with AM fungi, lime was the highest shoot dry weight followed by Troyer citrange. In the mycorrhizal plants with *P. parasitica*, shoot dry weight of Troyer citrange, Swingle citrumelo, lime and pomelo was lower than the mycorrhizal plants without *P. parasitica*, especially in the Swingle citrumelo and lime plants that had shoot dry weight significantly lower than the plants with *P. parasitica* (Table 3).

Root dry weight of non-mycorrhizal seedlings with and without *P. parasitica* did not have significantly difference. AM fungi significantly increased root dry weight of Troyer citrange, Swingle citrumelo and lime about 2 times compared with the non-mycorrhizal plants. Root dry weight of lime with AM fungi was highest followed by Troyer citrange and Swingle citrumelo, but root dry weight of Troyer citrange and Swingle citrumelo with AM fungi and *P. parasitica* was very low (Table 4).

### **Effect of AM fungi and citrus species as rootstocks on growth of the tangerine variety Sainamphung scions**

In the treatment without AM fungi, height of the scion of tangerine variety Sainamphung was lowest on the rootstock of Swingle citrumelo and highest on the rootstock of lime. However, AM fungi significantly increased height of the scion on rootstock of Swingle citrumelo nearly 4 times of the scion on the rootstock without AM fungi, and it was not significant difference with the scion on rootstock of lime with AM fungi. AM fungi increased dry weight of the scions on lime, pomelo, Swingle citrumelo and Troyer citrange rootstocks except Cleopatra tangerine. Dry weight of the scion of tangerine variety Sainamphung was highest on the lime rootstock (Table 5).

#### **Effect of AM fungi and citrus species as rootstocks on nutrient contents of the tangerine variety Sainamphung scions**

In the Table 6, the results showed that AM fungi did not have effect on N, P, K, Ca and Mg contents of the scions on the Cleopatra tangerine rootstocks. However, Cleopatra tangerine rootstock had efficient uptake Ca and Mg compared with the other citrus rootstocks. AM fungi significantly increased most of the nutrient contents of the tangerine variety Sainamphung scions on the Swingle citrumelo, lime and pomelo rootstocks. Nitrogen contents in the scions on the Swingle citrumelo and pomelo rootstocks with AM fungi significantly higher than both of the rootstocks without AM fungi. Especially, in the scion on the pomelo rootstock which had N content about 2.5 times of the pomelo rootstock without AM fungi. Phosphorus content in the scion on the lime rootstock inoculated with AM fungi was more than 4 times of the non-inoculated one. The scions of tangerine variety Sainamphung accumulated the highest phosphorus and potassium contents on the lime rootstock inoculated with AM fungi.



## Discussion

Although, AM fungi are obligate symbiosis of most terrestrial plant species (Smith and Read, 2008). However, different plant species had different responses to AM fungi (Requena, et al., 2001; Jifon et al., 2002; Youpensuk, 2006). Seedlings of Troyer citrange, Swingle citrumelo, lime and pomelo had shoot dry weight higher than the non-inoculated ones. Whereas, the citrus plants inoculated with AM fungi and *P. parasitica*, only lime seedlings had shoot dry weight significantly higher than the non-inoculated ones. However, shoot dry weight of lime inoculated with AM fungi and *P. parasitica* was lower than the one inoculated with only AM fungi because of the competition effect between AM fungi and *P. parasitica*. Lime was the best response to AM fungi it had the highest both shoot and root dry weight compared with the other citrus plants. The more amount of roots helped increasing of nutrient uptake for the growth of the shoots. Moreover, root colonization of AM fungi in lime was also very high about 87-89% causing of increasing efficiency of nutrient uptake to the host.

Plant species and varieties are also different resistance to plant pathogens. The results of this experiment showed that tangerine variety Sainamphung was most susceptible of the citrus plants without AM fungi and Cleopatra tangerine was the most resistant to *P. parasitica*. Colburn and Graham (2007) reported that Cleopatra tangerine rootstock was more resistant to root rot disease than Troyer citrange rootstock. In this experiment, AM fungi significantly reduced disease severity caused by *P. parasitica* of all the citrus plants. It is known that plants have their own immune system including physical and chemical barriers and several active mechanisms (Kachroo and kachroo, 2009). Interactions between plants and microbes result in plant disease or symbiosis. Plants detect both pathogenic and symbiotic microbes by a similar set of genes (Zhao and Qi, 2008).



Induced systemic resistance can be activated upon colonization of roots by nonpathogenic microbes (Van Loon et al., 1998). Many studies indicated that root colonization of AM fungi could reduce disease severity of pathogens via several mechanisms including induced disease resistance, increasing the nutrient uptake and plant growth (Sundaresan et al., 1993; Trotta et al., 1996; Ozgonen and Erkilic, 2007; Kapoor, 2008). Amount of zoospores of *P. parasitica* in pot experiment inoculated with AM fungi were lower than in the one without AM fungi. Especially in the pots of Cleopatra tangerine and lime, zoospores of *P. parasitica* in the pot inoculated with AM fungi were decreased more than 100 times of the pots without AM fungi. Reduction of zoospores of *P. parasitica* in the pots may due to the competition with AM fungi and resistance of the citrus plants inoculated with AM fungi.

Three months after grafting the scions of tangerine variety Sainamphung on the seven-month old seedlings of Cleopatra tangerine, lime, pomelo, Swingle citrumelo and Troyer citrange root stocks, the results showed that AM fungi significantly increased height of the scions of tangerine variety Sainamphung only on the root stock of Swingle citrumelo. Whereas, AM fungi significantly increased shoot dry weight of the scions on lime, pomelo, Swingle citrumelo and Troyer citrange root stocks except Cleopatra tangerine rootstock. The only height did not indicate the growth of plants because the plants can grow by increasing height and branches. The scion of tangerine variety Sainamphung grew best on the root stock of lime with AM fungi. It had the highest both height and shoot dry weight on the lime rootstock inoculated with AM fungi. AM fungi and citrus rootstocks also had different effect on accumulation of mineral contents of in the tangerine variety Sainamphung scions. AM fungi did not have significant effect on N, P, K, Ca and Mg contents of the tangerine variety Sainamphung scions on the rootstocks



of Cleopatra tangerine. In this study Cleopatra tangerine had root colonization of AM fungi about 47-49% that was lower than in the other citrus roots. Whereas, Swingle citrumelo, lime and Pomelo with AM fungi significantly increased of most nutrient contents of N, P, K, Ca and Mg in the tangerine variety Sainamphung scions compared with the non-mycorrhizal plants. Hyphae of AM fungi are both internal and external roots of the host plants. External hyphae of AM fungi associated with roots of the host plants increase mineral uptake to the host plants (Frey and Schuepp, 1993). AM fungi increased many kinds of nutrient contents according to the host plants and soil conditions (Marschner and Dell, 1994; Taylor and Harrier, 2001; Youpensuk et al., 2006). The tangerine variety Sainamphung scions on the lime rootstocks with AM fungi had P and K contents significantly higher than the scions on the other citrus rootstocks. Ca and Mg contents in were high in the tangerine variety Sainamphung scions on the rootstocks of Cleopatra tangerine with and without AM fungi. Pectic substances that cross-link with calcium in middle lamella become calcium pectate, which increase strength of plant cell resistance to plant pathogens. Moreover, Cytosolic  $\text{Ca}^{2+}$  in plant is a component of signals in resistance to plant pathogens (Scheel, 1998; Sanders et al., 2002; Strange, 2003). Therefore, the increase of Ca contents in plants causes increasing in disease resistance.

Conclusion of this research, Growth of lime seedling with AM fungi was highest, while growth of tangerine variety Sainamphung and Cleopatra tangerine was lowest. In the citrus seedlings without AM fungi, tangerine variety Sainamphung was the most susceptible and Cleopatra tangerine was the most resistance to *P. parasitica*. AM fungi increased resistance to *P. parasitica* of all citrus plants used in this research.

AM fungi increased growth of the scions of tangerine variety Sainamphung on the citrus rootstocks except on the Cleopatra tangerine rootstock. Lime with AM fungi was the best rootstock for growth of the tangerine variety Sainamphung scion.

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**Table 1** Effect of AM fungi on disease severity of *P. parasitica* in citrus plants

Citrus plant	Disease severity (%)	
	<i>P. parasitica</i>	AM fungi and <i>P. parasitica</i>
Sainamphung	54.5a	7.0d
Cleopatra	21.3c	1.3d
Troyer	50.0a	13.3cd
Swingle	31.1b	18.4c
Lime	49.5a	4.8d
Pomelo	32.7b	6.3d

Means followed by different letters are significantly different ( $P \leq 0.05$ ) by Duncan's Multiple Range Test.

**Table 2** Root colonization of AM fungi in citrus plants

Citrus plant	Root colonization of AM fungi (%)	
	AM fungi	AM fungi + <i>P. parasitica</i>
Sainamphung	69.1b	71.4b
Cleopatra	47.0c	49.0c
Troyer	64.5b	72.5b
Swingle	74.8ab	83.7a
Lime	86.7a	88.8a
Pomelo	72.1b	71.0b

Means followed by different letters are significantly different ( $P \leq 0.05$ ) by Duncan's Multiple Range Test.

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

citrus plant	Shoot dry weight of citrus plants (g)			
	Control	AM fungi	<i>P. parasitica</i>	AM fungi + <i>P. parasitica</i>
Sainamphung	0.6d	0.6d	0.6d	1.4cd
Cleopatra	0.7d	1.4cd	1.1cd	1.9cd
Troyer	0.8d	5.0ab	0.8d	2.8bcd
Swingle	1.6cd	4.3b	1.2cd	1.4cd
Lime	1.8cd	7.0a	1.5cd	4.8b
Pomelo	0.6d	3.3bc	1.0d	2.3bcd

Means followed by different letters are significantly different ( $P \leq 0.05$ ) by Duncan's Multiple Range Test.

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

Citrus plant	Root dry weight of citrus plants (g)			
	Control	AM fungi	<i>P. parasitica</i>	AM fungi + <i>P. parasitica</i>
Sainamphung	0.4d	0.8d	0.5d	1.6cd
Cleopatra	1.1d	1.1d	1.1d	1.3cd
Troyer	1.4cd	3.4ab	1.0d	1.2cd
Swingle	1.4cd	3.1abc	1.2cd	1.1d
Lime	2.3bcd	5.0a	2.0bcd	3.5ab
Pomelo	1.2cd	2.3bcd	1.7bcd	1.5cd

Means followed by different letters are significantly different ( $P \leq 0.05$ ) by Duncan's Multiple Range Test.

**Table 5** Effect of AM fungi and rootstocks of *Citrus* spp. on growth of the scions of tangerine variety Sainamphung

Citrus root stock	Growth of the scions of tangerine variety Sainamphung	
	Height of the scions (cm)	Dry weight of the scions (g)
Cleopatra	15.5bc	20.7c
Cleopatra + AM	18.2bc	20.7c
Troyer	18.5bc	21.0c
Troyer + AM	24.0ab	23.7b
Swingle	9.3c	16.8d
Swingle + AM	34.6a	24.3b
Lime	25.6ab	23.0b
Lime + AM	36.5a	26.9a
Pomelo	17.0bc	14.0e
Pomelo + AM	19.0bc	21.0c

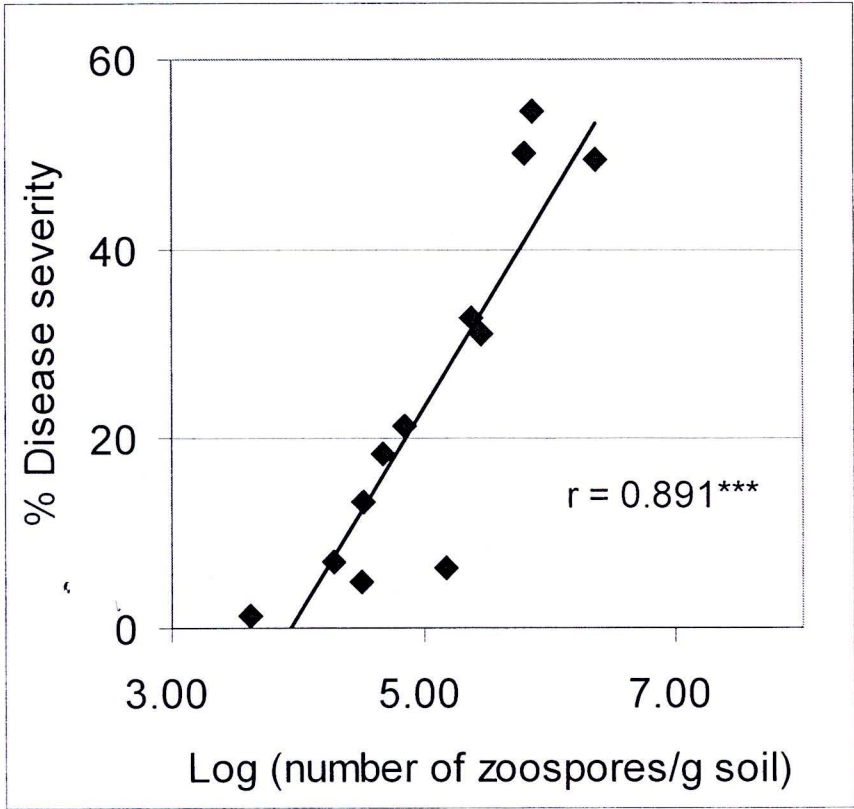
Means in the same column followed by different letters are significantly different ( $P \leq 0.05$ ) by Duncan's Multiple Range Test.

**Table 6** Effect of AM fungi and rootstocks of *Citrus* spp. on nutrient contents of the scions of tangerine variety Sainamphung

Citrus root stock	Nutrient contents of the scions of tangerine variety Sainamphung (mg/plant)				
	N	P	K	Ca	Mg
Cleopatra	496cd	38bc	360e	228ab	48ab
Cleopatra + AM	497cd	31cd	345e	238ab	52a
Troyer	502c	32cd	563bc	176de	30cd
Troyer + AM	505c	35bc	503d	208bcd	42b
Swingle	433d	26de	385e	149e	29cd
Swingle + AM	631a	42b	558cd	253a	45b
Lime	484cd	27de	608b	158e	30cd
Lime + AM	543bc	126a	789a	222abc	43b
Pomelo	231e	22e	269f	185cde	26d
Pomelo + AM	571ab	37bc	538cd	207bcd	35c

Means in the same column followed by different letters are significantly different ( $P \leq 0.05$ ) by Duncan's Multiple Range Test.





**Figure 1** Correlation between disease severity of the citrus plants and the amount of zoospores of *P. parasitica*.



