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Original article

Under sterilized and non-sterilized soil conditions, mycorrhizal dependency in citrus plants depends on phosphorus fertilization rather than zinc application

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Summary

The soils of the Çukurova Region (Eastern Mediterranean coast of Turkey) have high clay, pH and lime content; consequently, crop production is limited as zinc (Zn) and phosphorus (P) deficiency are common problems in citrus plantations. The objective of this study was to evaluate the effects of a single selected arbuscular mycorrhiza (AM) and indigenous mycorrhizae (IM) inoculations, using sterile and non-sterile soils, and the application of P and Zn doses on the growth and nutrient uptake of sour orange (*Citrus aurantium* L.) seedlings. Experiments were performed under greenhouse conditions using a Çanakçı soil series (Typic xerofluvent) over 8 months. *Rhizoglomus clarum* was used as the arbuscular mycorrhizae fungus at a level of 1000 spores per plant. Different P (0 and 200 mg kg^{-1} P_2O_5) and Zn (0, 2.5, 5 mg kg^{-1} Zn) rates were applied at the beginning of the experiment.

Generally, plants grown in non-sterile soils grew better than those in sterile soils. Mycorrhizal inoculation increased certain growth parameters, such as shoot height, shoot diameter, shoot and root dry matter, and root colonization in not only sterile but also non-sterile soils. However, mycorrhizal inoculation in sterile soil significantly increased plant biomass and nutrient uptake. The results show that the non-sterile soil significantly increased citrus dry matter production, % root colonization, and P and Zn uptake compared to sterile soil. In non-inoculated, sterile soils, plant P and Zn content was significantly reduced compared with inoculated plants. In addition, in sterile soil, the seedling P and Zn concentration increased upon mycorrhizal inoculation. Inoculation dependency was significantly affected by soil sterilization and partly by P application but not by Zn application. Under non-sterile soil conditions, since there was native mycorrhiza spores mycorrhizal dependency (MD) was very low and also increasing P addition have reduced mycorrhizal dependency.

Keywords
sour orange, mycorrhizal dependency, soil sterilization, phosphorus and zinc uptake

Significance of this study

What is already known on this subject?

- The sour orange/citrus plant is extremely dependent on mycorrhiza. Mycorrhizal dependency is mainly based on phosphorus nutrition. However, the effect of zinc nutrition on mycorrhizal dependency is less known. The aim of the study was to determine the effects of selected arbuscular mycorrhizae (AM) and indigenous mycorrhizae (IM) inoculations with application of P and Zn doses on the growth and nutrient uptake of sour orange (*Citrus aurantium* L.) seedlings in sterile and non-sterile soils.

What are the new findings?

- Sour orange/citrus plants were better able to grow without mycorrhizae inoculation in non-sterile soil with the help of indigenous mycorrhizae. In present experiment we tried to clarify the effect of P and Zn nutrition on mycorrhizal dependency. We found that under non-sterile soil conditions, increasing Zn application decreased mycorrhizal dependency.

What is the expected impact on horticulture?

- Mycorrhiza has a great importance for sustainable agriculture, which enhances the growth of citrus plants/sour orange especially in the soils with P and Zn deficiency. The results are strongly suggesting that mycorrhizal inoculation is necessary for better plant growth even under non-sterile conditions.

Introduction

Citrus plants are commonly grown in the Çukurova Region (in particular, the Eastern Mediterranean coast of Turkey). Due to high pH and calcium carbonate ($CaCO_3$) the soil fertility and P and Zn availability of Çukurova soils is limited (Ortas et al., 2002). Arbuscular mycorrhizal (AM) symbiosis is a mutualistic association between fungi and the roots of terrestrial plants, which is suggested to be a natural solution to increase crop productivity in developing countries. Citrus roots normally form symbiotic associations with arbuscular mycorrhizal fungi (AMF) (Rayner, 1935) and citrus plants require mycorrhizal colonization for maximum growth (Graham and Syvertsen, 1985; Menge et al., 1982).

The health of the citrus plant and degree of AM fungal

colonization have been found to be related; the healthiest plants were the most highly colonized (Michelini et al., 1993). Mycorrhizae fungi can increase the rates of seedling survival and plant growth. Recently, horticultural seedling industries have begun to recognize the importance of mycorrhizae and offer products which inoculate plant roots with mycorrhizal fungi (Ortas, 2012). Within mycorrhizal associations, plants supply labile photosynthates to fungi, while fungi aid in the uptake of nutrients, especially P. The potential of AMF to increase citrus plant growth under conditions of low soil P has been well documented (Ortas, 2012).

Ortas et al. (2016) reported that plant growth was reduced as a result of soil sterilization due to the elimination of viable mycorrhiza. Masri et al. (1998) reported that *Funneliformis mosseae* enhanced growth, leaf transpiration and conductance of *Garcinia mangostana* L. and also caused a 67–88% improvement of P uptake efficiency. Mycorrhizal inoculation has many effects on horticultural plants, including increasing citrus seedling survival rate and growth (Ortas et al., 2016). Also, it has been reported that AMF-inoculated citrus seedlings exhibited higher total root length, total root surface area and total root volume (Wu et al., 2011).

As citrus plants have coarse root systems, they mainly depend upon mycorrhizal colonization in order to obtain sufficient nutrients and water; hence, plant species such as citrus are obligately mycorrhizal dependent (Graham, 1986; Graham and Syvertsen, 1985; Levy and Krikun, 1980; Menge et al., 1982; Ortas et al., 2016). Sour orange inoculated with *Rhizophagus clarum* has been shown to significantly increase plant growth (Ortas et al., 2002; Wu and Xia, 2004, 2005).

Under orchard conditions citrus roots are usually colonized by mycorrhiza (Nemec et al., 1981). However, under heavy agrochemical, irrigation and tillage treatments, the mycorrhizal colonization ratio is reduced. Under these conditions, it is important to use seedlings inoculated with mycorrhizal fungi.

Sour orange (*Citrus aurantium* L.) is the most commonly used rootstock due to lower cost in the Mediterranean regions. Most citrus species, such as sour orange, trifoliate orange *Cleopatra mandarin*, swingle citrumelo and 'Carrizo' citrange, have few short root hairs and are heavily dependent on AMF (Davies and Albrigo, 1994; Graham and Syvertsen, 1985). As citrus plants depend on mycorrhizal colonization (Menge et al., 1978; Ortas, 2012), AMF can improve plant growth and nutrient uptake, especially P and Zn (Graham and Syvertsen, 1985; Ortas et al., 2016). In the heavy clay soil of the Mediterranean, *R. clarum* proved to be one of the most effective mycorrhizal fungi for sour orange, in terms of mycorrhizal colonization, and growth and nutrient uptake (Ortas et al., 2002). Fidelibus et al. (2001) reported that although the greatest differences were between AM and non-AM citrus plants, plants treated with *Glomus* isolates differed in colonization level, leaf P concentration, root length, transpiration flux and leaf conductance. The AMF *R. intraradices* was shown to increase the root and shoot growth of the citrus rootstocks sour orange (*Citrus aurantium* L.) and 'Carrizo' citrange (*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.) (Dutra et al., 1996).

As citrus seedlings with small root systems are vulnerable to desiccation (Davies and Albrigo, 1994), the inoculation of citrus roots with AMF populations that enhance growth might improve the transplant survival of seedling nursery stock into field sites. Suppression of root growth might result from increased AMF activity leading to higher carbon costs to the plant (*Citrus volkameriana*) (Fidelibus et al., 2000).

This study was based on the hypothesis that under sterile and non-sterile conditions mycorrhizal inoculation increases sour orange seedling growth and nutrient uptake. The objective of the study was to evaluate the effects of different rates of P and Zn application on sour orange growth and nutrient uptake, under sterile and non-sterile soil conditions, with and without mycorrhizal inoculation.

Materials and methods

Experiments were conducted under greenhouse conditions in the University of Çukurova, Soil Science and Plant Nutrition Department, Adana, Turkey. The Çanakçı soil series (Typic xerofluvent) was sterilized for 2 h at 120°C in an autoclave for use as a growth medium. The initial properties of the non-autoclaved soil are presented in Table 1.

Production of seedlings

Sour orange (*Citrus aurantium* L.) seeds were surface sterilized with a sodium hypochlorite solution (1% available chlorine) for 10 minutes, rinsed and then soaked in distilled water four times. Seeds were grown for 5 weeks in perlite trays and grown under glasshouse conditions; when the seedlings reached the 3-leaf stage they were transplanted into plastic pots containing 3 kg of soil.

Inoculum source

Plants were inoculated at transplantation stage with *Rhizoglomus clarum* (T.H. Nicolson & N.C. Schenck) Sieverd., G.A. Silva & Oehl (Sieverding et al., 2014). Thousand spores were applied per seedling, located 50 mm each the seedling. In the non-mycorrhizal treatments, each seedling received the same amount of mycorrhiza-free substrate (autoclaved growing medium). In order to reintroduce the soil organisms such as bacteria (apart from mycorrhiza fungi) into the sterilized soil, rhizosphere soil solution was filtered (10 μ) from the experimental soil, followed by incubation for two weeks prior to being used. *R. clarum* was propagated using maize as the host plant.

Table 1. Selected physical, chemical and biological properties of Çanakçı soil series.

Properties	Unit		
Clay	g kg^{-1}	288	±0
Silt		590	±0
Sand		122	±0
Soil organic carbon	g kg^{-1} soil	0.84	±0.10
Inorganic carbon		2.8	±0.15
Total nitrogen		0.19	±0.01
CEC*	$Cmol^{+}$ kg^{-1}	20.50	±2.00
pH	H_2O	7.52	±0.50
Salt	%	0.06	±0.12
P_2O_5	kg ha^{-1}	7.10	±0.90
K_2O		153	±2.83
Fe		3.84	±0,40
Mn		2.14	±0.31
Zn		0.40	±0.13
Cu		1.46	±0.20
Number of AMF spores	10 g^{-1} soil	62	±18

Values are the averages of three samples ± standard deviation.
*CEC: Cation Exchange Capacity.

Table 2. The effects of different levels of P (mg P kg^{-1}) and Zn (mg Zn kg^{-1}) application and mycorrhizal inoculation on root colonization (%) under sterile and non-sterile soil conditions.

Treatments		Root colonization (%)	
		Non-sterile	Sterile
(-M)	P0 Zn 0	40 ±36 B	2 ±4 C
	P0 Zn 1	43 ±12 B	4 ±3 C
	P0 Zn 2	80 ±17 A	0 ±0 D
	P1 Zn 0	60 ±17 AB	0 ±0 D
	P1 Zn 1	47 ±15 AB	3 ±8 C
	P1 Zn 2	57 ±12 AB	0 ±0 D
	Mean	55 A	2 B
(+M)	P0 Zn 0	63 ±21 AB	60 ±20 AB
	P0 Zn 1	80 ±10 A	51 ±10 AB
	P0 Zn 2	50 ±17 AB	60 ±17 AB
	P1 Zn 0	60 ±30 AB	40 ±26 B
	P1 Zn 1	43 ±31B	33 ±15 B
	P1 Zn 2	40 ±26 B	63 ±15 AB
	Mean	56 A	51 A

Mean of three replicates and ± is standard error.
Columns with different letters are significantly different ($P < 0.05$) according to LSD test.

Experimental design

At the beginning of the experiment, sterile and non-sterile soils were treated with two levels of P, 0 and 200 mg P_2O_5 kg^{-1} soil (P0-P1), and three levels of Zn, 0, 2.5, 5 mg kg^{-1} soil (Zn0-Zn1-Zn2). The experiment was completely randomized and three replicates were used. Once the seedlings were transplanted, the plantlets were grown for 8 months in a controlled glasshouse with day-night temperatures of 26 ± 3°C. Plantlets were maintained under a 16 h photoperiod, using cool white fluorescent lamps, at approximately 350 µmol m^{-2} s^{-1}. The relative humidity was 70 to 80% at night and 80 to 85% during the day. Distilled water was added daily to maintain the moisture at 75% of field capacity after pots were weighed daily.

Dry matter assessment and nutrient analysis

At harvest, the shoot and root dry weight (in g $plant^{-1}$) were measured for each treatment. The shoots were separated from the roots 0.5 cm above the soil surface, and the roots were separated from the soil by washing with running tap water and distilled water and dried on tissue paper. A sub-sample of the root was reserved for root colonization analyses and the remainder of the roots were dried at 70°C for 48 hours. After shoot and root material were dried they were then ground using a Tema mill. Ground plant material was ashed at about 550°C, and the residue was extracted with 3.3% HCl. The concentration of P was determined according to Murphy and Riley (1962) using a flame photometer. An ICP (Perkin Elmer) was employed to determine the Zn concentration.

Mycorrhizal colonization (%)

Small sub-samples from the roots were preserved in a mixture of ethanol, glacial acetic acid and formalin for determination of root length and mycorrhizal colonization. Roots were stained as described by (Koske and Gemma, 1989) and determined by the method of Giovannetti and Mosse (1980).

Mycorrhizal dependency

After harvest, the total dry weight of the seedlings was recorded and inoculation mycorrhizal dependency of the seedlings by AMF was calculated using the following formula:

$$Mycorrhizal\ dependency\ (MD) = \frac{\text{TDW (+M)} - \text{TDW (−M)}}{\text{TDW (+M)}} \times 100$$

TDW = total dry weight; +M = inoculated seedlings; -M = non-inoculated seedlings.

Statistical analysis

The variation in plant parameters was assessed via the analysis of variance ANOVA procedure using the SPSS 10.0 for Windows statistical analysis programme. The main significant interactive effects of mycorrhizal inoculation and growth medium were separated using the LSD test at $P < 0.05$.

Results and discussion

Root colonization

Root colonization was statistically significantly affected by soil sterilization, mycorrhiza and the sterilization–mycorrhizae interaction (Table 3). In non-sterile soil, the indigenous colonization ranged from 40 to 80% (with a mean of $\bar{X}$=55 and $\bar{X}$=56). In sterile soil without mycorrhizal inoculation, the per cent root colonization ranged from 0 to 4%, with a mean of $\bar{X}$=2; however, in mycorrhiza-inoculated treatments, the root colonization ranged from 33 to 60%, with a mean of $\bar{X}$=51.

Increasing the addition of P and Zn did not make any significant change to root colonization levels (Table 3). Graham et al. (1991) reported that 'Volkamer' lemon, which, like many species, are highly dependent on the root colonization when grown in low P soil, maintains relatively high rates of the root colonization even at high P supply.

Shoot and root fresh and dry weight

In non-sterile soils, due to the potential indigenous mycorrhiza propagules there were no differences between the fresh weights of the inoculated and non-inoculated plants. However, in sterile soils there were significant differences between the inoculated and non-inoculated treatments. In the non-inoculated sterile soil, the shoot fresh weight (SFW) ranged from 0.2 g pot^{-1} to 3.7 g pot^{-1}, with a mean of $\bar{X}$=1.8 g pot^{-1}. In mycorrhiza-inoculated sterile soil, the SFW ranged from 22.0 g pot^{-1} to 35.3 g pot^{-1}, with a mean of $\bar{X}$=31.5 g pot^{-1} (Table 4).

In non-sterile soil, there was no difference in the shoot dry weight (SDW) between non-inoculated and inoculated citrus plants. In non-sterile soils, with no inoculation of the citrus plant, the SDW ranged from 11.2 g pot^{-1} to 16.3 g pot^{-1}. In non-sterile soil with mycorrhizal inoculation, the SDW ranged from 11.8 g pot^{-1} to 18.0 g pot^{-1}. In sterile soil with no inoculation, the SDW ranged from 0.1 g pot^{-1} to 1.7 g pot^{-1}, with a mean of $\bar{X}$=0.9 g pot^{-1}. Furthermore, mycorrhizal inoculation resulted in significantly higher SDW than non-mycorrhizal inoculation in sterile soil. With mycorrhizal inoculation, the SDW ranged from 10.6 g pot^{-1} to 17.1 g pot^{-1}, with a mean of $\bar{X}$=15.2 g pot^{-1} (Table 4).

Root fresh and dry weights also differed with soil sterilization (Table 4). In non-sterile soil there were no differences between the non-inoculated and inoculated treatments. However, in sterile soil, the root, fresh and dry weight increased significantly upon mycorrhizal inoculation compared with the non-inoculated treatment. In sterile soil, in

Table 3. Significance of F-values (probability) from analysis of variance for different plant parameters under soil sterilization, mycorrhizal inoculation, phosphorus and zinc application.

Treatments	DF	SFW	SDW	RFW	RDW	TDW	P %	Zn	MRC
Sterile (S)	1	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0050	<.0001
Mycorrhiza (M)	1	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
S × M	1	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Phosphorus (P)	1	0.0073	0.0030	0.0703	0.0022	0.0014	<.0001	0.2307	0.1520
S × P	1	0.8117	0.8555	0.5704	0.5472	0.7090	<.0001	0.8459	0.7722
M × P	1	0.7074	0.7555	0.2898	0.6885	0.7046	0.4230	0.3089	0.1868
S × M × P	1	0.8819	0.9130	0.4018	0.3807	0.7323	0.5473	0.5483	0.6854
Zinc (Zn)	2	0.0184	0.0001	0.0012	0.0159	0.0022	0.8900	0.0951	0.4537
S × Zn	2	0.0411	0.1209	0.5644	0.4014	0.1953	0.1798	0.1675	0.7977
M × Zn	2	0.8632	0.9794	0.6329	0.8382	0.9833	0.1168	0.2803	0.4391
S × M × Zn	2	0.0589	0.0483	0.4086	0.6015	0.1825	0.3669	0.9991	0.0355
P × Zn	2	0.2928	0.5483	0.0009	0.0787	0.0018	0.2334	0.3949	0.7360
S × P × Zn	2	0.0335	0.2497	0.9717	0.6189	0.3803	0.9001	0.0544	0.2500
M × P × Zn	2	0.4471	0.6297	0.0053	0.8806	0.2913	0.7352	0.7133	0.6398
S × M × P × Zn	2	0.3577	0.2997	0.4306	0.4313	0.4106	0.2586	0.2336	0.3340

SFW: Shoot fresh weight; SDW: Shoot dry weight; RFW: Root fresh weight; RDW: Root dry weight; TDW: Total dry weight; P: % tissue P concentration; Zn: Zn concentration mg Zn $kg^{-1;}$ MRC: mycorrhizal root colonization %.

Table 4. The effect of the different levels of P (mg P kg^{-1}) and Zn (mg Zn kg^{-1}) soil sterilization and mycorrhizal inoculation applied to the soil on shoot and root weights of citrus plants (g pot^{-1}).

		Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight	Total dry weight	Shoot/Root dry ratio
				(Non-sterile)			
(-M)	P0 Zn 0	23.8±0.2 B-D	11.2±0.2 EF	15.5±1.1 DE	8.7±0.4 DC	19.9±0.9 CD	1.2
	P0 Zn 1	30.5±3.2 A-D	14.1±2.12 BD	18.3±2.8 B-D	10.1±1.6 A-C	24.2±3.7 A-C	1.4
	P0 Zn 2	34.0±3.5 AB	15.7±1.5 A-C	21.0±1.9 A-C	11.3±0.7 A-C	27.0±4.5 A	1.4
	P1 Zn 0	31.0±2.7 A-D	14.9±2.1 B-D	18.4±5.2 B-D	11.1±3.4 A-C	25.4±5.5 AB	1.3
	P1 Zn 1	30.4±1.2 A-D	13.9±0.8 C-E	23.1±3.0 AB	12.2±1.9 AB	26.1±2.3 AB	1.1
	P1 Zn 2	33.9±3.8 AB	16.3±2.1 A	21.1±1.6 A-C	11.4±1.2 AB	27.7±0.8 A	1.4
	Mean	30.6A	14.2 A	19.6 A	10.8 A	25.0 A	1.3
(+M)	P0 Zn 0	21.2±1.6 D	11.8±0.9 DF	15.2±2.9 DE	9.6±1.7 D	21.4±1.1 B-D	1.4
	P0 Zn 1	34.3±1.6 A	16.4±0.4 AC	20.3±1.1 A-C	11.1±0.9 A-C	27.4±1.0 A	1.5
	P0 Zn 2	31.9±5.3 A-C	18.0±3.1 AC	20.7±3.3 A-C	11.9±4.1 A-B	27.4±6.9 A	1.5
	P1 Zn 0	31.4±2.2 A-C	15.0±0.6 A-C	17.8±3.0 CD	11.4±1.3 ACD	26.4±2.7 A	1.3
	P1 Zn 1	34.9±2.3 A	16.6±1.4 AB	18.9±1.0 A-D	11.0±0.2 A-C	27.7±1.4 A	1.5
	P1 Zn 2	31.7±4.9 A-C	16.0±2.1 A-C	17.4±4.3 CD	11.9±1.9 A-D	27.5±2.0 A	1.5
	Mean	30.9 A	15.2 A	18.4 A	11.1 A	26.3 A	1.4
				(+Sterile)			
(-M)	P0 Zn 0	0.2±0.0 E	0.1±0.0 G	0.3±0.0 F	0.1±0.1 E	0.2±0.1 C	0.7
	P0 Zn 1	0.3±0.0 E	0.1±0.0 G	0.6±0.1 F	0.1±0.0 E	0.2±0.01 E	0.9
	P0 Zn 2	0.2±0.1 E	0.1±0.1 G	0.5±0.1 F	0.1±0.0 E	0.2±0.1 E	1.4
	P1 Zn 0	3.3±1.0 E	1.5±0.2 G	3.2±0.7 F	1.7±0.5 E	3.1±0.8 E	0.7
	P1 Zn 1	3.2±1.0 E	1.8±0.5 G	2.3±0.7 F	1.3±0.4 E	3.0±0.8 E	1.4
	P1 Zn 2	3.7±1.58 E	1.7±0.4 G	2.4±0.7 F	1.5±0.7 F	3.1±1.1 E	1.1
	Mean	1.8 B	0.9 B	1.5 B	0.8 B	1.6 B	1.16
(+M)	P0 Zn 0	22.0±3.1 D	10.6±1.4 F	11.7±1.3 E	7.4±1.2 D	18.0±2.6 D	1.44
	P0 Zn 1	31.8±1.3 A-C	16.4±1.9 A-C	23.1±2.2 AB	12.2±1.9 AB	28.7±0.6 A	1.35
	P0 Zn 2	35.3±1.2 A	17.1±0.8 A-C	23.6±5.2 A	12.0±0.6 AB	29.1±1.2 A	1.43
	P1 Zn 0	30.7±6.7 A-D	14.8±3.6 A-C	22.2±1.4 A-C	12.1±0.8 AB	26.9±5.3 AB	1.22
	P1 Zn 1	33.9±4.3 AB	15.4±3.5 A-C	22.0±1.8 A-C	13.7±2.2 A	27.7±6.6 A	1.12
	P1 Zn 2	35.1±2.8 A	16.7±1.2 AB	19.4±3.0 A-D	12.1±1.3 AB	28.9±1.8 A	1.38
	Mean	31.5A	15.2 A	20.3 A	11.6 A	26.8 A	1.33

Mean of three replicates and ± is standard error.
Columns with different letters are significantly different ($P < 0.05$) according to LSD test.

Table 5. The effect of the different levels of P (mg P kg^{-1}) and Zn (mg Zn kg^{-1}) soil sterilization and mycorrhizal inoculation applied to the soil on P (%) and Zn (mg kg^{-1} SDW) concentration of SDW in citrus plant.

Treatments		P	Zn
		Non-sterile	
(-M)	P0 Zn 0	0.08 ±0.01 D-F	17.80 ±4.95 C-F
	P0 Zn 1	0.13 ±0.00 CD	21.30 ±1.50 A-D
	P0 Zn 2	0.12 ±0.00 CD	23.17 ±2.74 A-C
	P1 Zn 0	0.21 ±0.02 A	22.77 ±4.53 A-C
	P1 Zn 1	0.22 ±0.08 A	23.20 ±5.80 A-C
	P1 Zn 2	0.19 ±0.03 AB	23.27 ±4.97 A-C
	Mean	0.16 A	22.25 A
(+M)	P0 Zn 0	0.08 ±0.01 D-F	18.22 ±0.70 C-F
	P0 Zn 1	0.11 ±0.02 DE	24.37 ±3.31 A-C
	P0 Zn 2	0.12 ±0.01 D	25.27 ±2.61 A-C
	P1 Zn 0	0.19 ±0.07 AB	20.60 ±0.66 A-E
	P1 Zn 1	0.19 ±0.01 AB	21.67 ±3.56 A-D
	P1 Zn 2	0.22 ±0.08 A	24.90 ±0.36 A-C
	Mean	0.15 A	22.51 A
		Sterile	
(-M)	P0 Zn 0	0.01 ±0.01 G	11.45 ±4.04 FG
	P0 Zn 1	0.06 ±0.05 E-G	13.30 ±6.02 E-G
	P0 Zn 2	0.01 ±0.01 G	9.78 ±4.88 G
	P1 Zn 0	0.03 ±0.00 FG	14.57 ±0.70 D-G
	P1 Zn 1	0.03 ±0.00 FG	10.17 ±8.01 G
	P1 Zn 2	0.03 ±0.01 FG	19.00 ±6.35 B-E
	Mean	0.03 B	13.05 B
(+M)	P0 Zn 0	0.10 ±0.01 DE	17.69 ±0.91 C-F
	P0 Zn 1	0.14 ±0.01 B-D	26.13 ±1.55 AB
	P0 Zn 2	0.13 ±0.01 CD	26.30 ±1.45 AB
	P1 Zn 0	0.18 ±0.02 A-C	23.67 ±3.10 A-C
	P1 Zn 1	0.14 ±0.01 B-D	24.37 ±3.60 A-C
	P1 Zn 2	0.13 ±0.02 CD	28.13 ±1.96 A
	Mean	0.14 A	24.38 A

Mean of three replicates and ± is standard error.
Columns with different letters are significantly different ($P < 0.05$) according to LSD test.

the non-inoculated treatment, the root dry weight (RDW) ranged from 0.1 g pot^{-1} to 1.7 g pot^{-1}, with a mean of $\bar{X}$=0.8 g pot^{-1}. In mycorrhiza-inoculated plants, the RDW ranged from 7.4 g pot^{-1} to 13.7 g pot^{-1}, with a mean of $\bar{X}$=11.6 g pot^{-1}. Increasing the application of Zn and P also increased the root fresh and dry weight.

The total dry weight (TDW) was significantly affected by soil sterilization, mycorrhizal inoculation, P and Zn addition (Table 3). As citrus plants are mycorrhiza dependent, and soil sterilization kills off all the indigenous mycorrhizal fungi, plants grown in sterile soil do not grow very well compared with plants grown in non-sterile soils. Higher plant growth in the non-sterile soil is due to the indigenous mycorrhizae significantly colonizing the plant roots. The work of Ortas (2012) and Ortas et al. (2002) showed that without mycorrhizal inoculation the plants grew better in non-sterile soil than in sterile soil, which can be attributed to the effectiveness of the indigenous mycorrhizae.

In sterile soils, mycorrhiza-inoculated plants exhibited a higher shoot/root ratio, with a mean of $\bar{X}$=1.3. Statistically, the fresh and dry shoot and root growth of the seedlings were significantly affected by soil sterilization, mycorrhiza and P treatment (Table 3). It seems that Zn application does not significantly affect citrus seedling growth.

Ortas (2012) also reported that the growth of mycorrhizae-inoculated citrus seedlings in P-deficient soil was higher than those of non-AM infected seedlings.

Plant nutrient status

In the overall, 4-way ANOVA analyses the % P of the citrus seedlings was significantly affected by soil sterilization, mycorrhiza and P treatment (Table 3). Mycorrhizal inoculation also significantly affected the plant P and Zn content (Table 3). In non-sterile soils, the plant P percentage ranged from 0.08% to 0.22%, with a mean of $\bar{X}$=0.16% P without mycorrhizal inoculation; with mycorrhizal inoculation, the range was 0.08% to 0.22%, with an average mean of $\bar{X}$=0.15% P (Table 5). In sterile soil, the percentage plant P ranged from 0.01% to 0.06%, with an average mean of $\bar{X}$=0.03% P without mycorrhizal inoculation; with mycorrhizal inoculation, the range was 0.10% to 0.18%, with an average mean of $\bar{X}$=0.14% P.

Generally, plants grown in non-sterile soil exhibited a higher % P content than those grown in sterile soil, and mycorrhizal inoculation resulted in a higher % P content (Table 5).

For both sterile and non-sterile soil, increasing the Zn concentration led to increased seedling Zn concentration. The Zn concentration of seedlings was significantly affected by soil sterilization, mycorrhiza and the sterilization–mycorrhizae interaction (Tables 3 and 5). Citrus seedlings grown in non-sterile soils, without mycorrhizal inoculation, contained a mean of 22.25 mg Zn kg^{-1} DW and with mycorrhizal inoculation, the mean was 22.51 mg Zn kg^{-1} DW. In non-sterile soils there were no big differences between treatments, however, in sterile soil, the Zn concentration of the mycorrhiza-inoculated plants was nearly double the level found in non-inoculated plants.

Mycorrhiza-inoculated sour orange plants produced a high Zn content, which exceeded the critical level (>20 mg Zn kg^{-1} DW). However, in sterile soil with no mycorrhizal inoculation the plant leaves Zn concentration was lower than 20 mg Zn kg^{-1} DW. Seedlings grown in sterile soils, without mycorrhizal inoculation contained a mean of 13.05 mg Zn kg^{-1} DW; however, mycorrhiza-inoculated plants contained a mean of 24.38 mg Zn kg^{-1} DW.

In this study, mycorrhizal inoculation with *R. clarum* was observed to be highly effective. Mycorrhiza-treated plants exhibited better plant growth and uptake of nutrients, such as P and Zn, in sterile soil conditions compared with non-sterile conditions. P and Zn concentrations in non-sterile soils were higher than in sterile soils due to the beneficial effects of mycorrhizae and other microorganisms. Ova et al. (2015) reported that under non-sterile soil conditions with high P application since native mycorrhizae are depressed plant root and tissue have Zn deficiency. Previously, Ortas et al. (2002) reported that *R. clarum*-inoculated sour orange seedlings exhibited an increased uptake of P and Zn in plants grown in sterile soils. Srivastava et al. (2002) reported that mycorrhizal-treated citrus trees exhibited better plant growth and uptake of nutrients, such as P and Zn, compared with non-mycorrhizal trees. In addition, it has been previously shown that the main effect of AM on plants is enhanced P absorption (Chen et al., 2014).

Although P application in sterile soil increased the plant

shoot and root dry matter compared with the P0 application, the influence of P addition had less effect than mycorrhizal inoculation. Chen et al. (2014) reported that *R. intraradices*-inoculated orange seedlings showed increased plant growth and leaf Zn concentration. As mycorrhizae and other microorganisms have beneficial effects, it can be concluded that it is very important to manage the mycorrhizal potential of indigenous soil in which citrus plants are grown. The effects of mycorrhiza in orchards are almost exclusively beneficial. Many experiments have shown that citrus plants are heavily dependent upon mycorrhizae (Yang et al., 2015). Results have shown that if the soil has enough mycorrhizal spores the plants may not need re-inoculation. In previous experiments we found that indigenous mycorrhizae also significantly inoculated citrus roots (Ortas and Ustuner, 2014). Finally, as can be seen from the tables it appears that the level of indigenous mycorrhiza in the non-sterile soil is sufficient to colonize the citrus plant compared with the level achieved after mycorrhizal inoculation. However, sterile soil definitely requires mycorrhizal inoculation for better plant growth. Organisms within the rhizosphere, especially indigenous mycorrhizae, may have some other benefit, apart from nutrient uptake, such as water uptake and disease control. In addition, indigenous inoculation, involving several mycorrhizal species, may significantly contribute to plant growth and nutrient uptake. Previously, Ortas and Ustuner (2014) used several mixed and dual-inoculated species and they found that dual species inoculation and indigenous mycorrhizae showed good potential for the production of healthy seedlings, in several growing media. Similarly, Santos et al. (2008) reported that the benefits of mycorrhizal fungi varied according to isolates and plant species.

Over all there is a significant difference in seedlings growth in between sterilized and non-sterilized soils. In non-sterilized soil, since there are living organisms including mycorrhizal spores, plant growth was not compromised. However, with soil sterilization, not only indigenous mycorrhizae were eliminated, but, may be significant substantial changes such as soil microflora, soil enzymes, aggregate and soil chemical characters influenced. Usually after soil sterilization ammonium and manganese (Mn) concentration increases and sometimes Mn concentration can reach to the phytotoxic levels. Since after autoclaving, soil nutrient concentration was not measured, we are not able to do more interpretations. For the next work, the effects of plant growth medium sterilization need to be searched extensively.

Mycorrhizal dependency

For both sterilized and non-sterilized soils, the MD was higher in P0 treated soils than P1 treated soils (Table 6). The MD of seedlings grown in sterilized soils was significantly higher than plants grown in non-sterilized soils. The non-sterile and the P0Zn0 treatment exhibited a 6.6% dependency, however the P1Zn0 treatment had a dependency of 3.7%. In sterile soils, P0Zn0 treated seedlings had an MD of 98.8%; on the other hand, the P1Zn0 treated seedlings had an MD of 880 %. The results are supporting our previous works (Ortas, 2012; Ortas et al., 2016) and since native mycorrhizal spores were eliminated under sterile soil conditions, plants strongly depending mycorrhizae for better P and Zn uptake.

Conclusions

Without mycorrhizal inoculation, seedlings grown in non-sterile soils grew much better than in sterile soils. In sterile soil, *R. clarum* inoculation had a significant effect on the growth and P and Zn concentration. Also indigenous mycorrhiza appears to have a significant impact on citrus seedling growth. In addition, soil sterilization significantly affected mycorrhizal dependency. Citrus plant strongly dependent on mycorrhizal inoculation rather than phosphorus and zinc fertilizer addition. P addition had effects on MD, however Zn application had no significant effects on MD. Possibly with soil sterilization, not only indigenous mycorrhizae were eliminated, may be significant substantial changes such as soil microflora, soil enzymes, aggregate and soil chemical characters such as ammonium, phosphorus, manganese, and soil pH influenced. The effects of plant growth medium sterilization need to be searched extensively. Further work needs to be performed to determine the effects of indigenous and selected mycorrhizae and their dual inoculation under greenhouse and field conditions.

Table 6. The effect of the different levels of P (mg P kg^{-1}) and Zn (mg Zn kg^{-1}) applied to total dry weight mycorrhizae dependency (%) of citrus seedlings.

Treatments		Mycorrhizal dependency
Non-sterile	P0 Zn 0	6.6
	P0 Zn 1	11.8
	P0 Zn 2	1.7
	P1 Zn 0	3.7
	P1 Zn 1	5.7
	P1 Zn 2	-0.7
Sterile	P0 Zn 0	98.8
	P0 Zn 1	99.5
	P0 Zn 2	99.4
	P1 Zn 0	88.0
	P1 Zn 1	89.0
	P1 Zn 2	89.2

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