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Physiological Responses of Sweet Basil (*Ocimum basilicum* L.) to Triple Inoculation with *Azotobacter*, *Azospirillum*, *Glomus intraradices* and Foliar Application of Citric Acid

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ABSTRACT

In order to study the effect of biofertilizers including mycorrhiza, Azotobacter and Azospirillum, and foliar spray of citric acid on vegetative traits of basil (Ocimum basilicum L.) a factorial experiment was conducted in the form of a randomized complete block design with four replications. Before planting, seeds were inoculated with mycorrhiza, Azotobacter and Azospirillum. Citric acid was applied as a foliar spray at a rate of 5 mM at 5-6 leaf stage. Results showed that foliar spray of citric acid significantly increased shoot fresh weight, shoot dry weight, root fresh weight and root dry weight. Mycorrhiza, Azotobacter and Azospirillum had no significant effect on these traits. Three-fold interaction of citric acid × Azotobacter × mycorrhiza had significant effect on the studied traits. Citric acid × Azotobacter × mycorrhiza produced the highest amount of shoot fresh weight, while the highest shoot dry weight was produced by citric acid × azospirillum. Citric acid × mycorrhiza produced the highest root fresh weight, whereas the highest root dry weight was produced by Azotobacter × mycorrhiza.

Keywords: *Azospirillum*, *Azotobacter*, Mycorrhiza, Organic acid.

INTRODUCTION

The *Ocimum* genus belonging to the Lamiaceae family is characterized by a great variability of both morphology and chemotypes [14]. This genus has more than 30 species [28]. Among the species of this genus, *Ocimum basilicum* has the most economic importance and is grown and utilized throughout the world [14]. *O. basilicum* is an annual plant [15, 18], originated from the North-West India, North-East Africa and Central Asia [3]. It is a valuable medicinal plant that not only has many applications in food, pharmacy, dentistry, perfumery and cosmetic industry, but also used extensively in traditional and modern medicine [18] and traditionally consumed as a medicinal herb to treat headaches, coughs, diarrhea, constipation, kidney disorders and parasitic diseases. In addition, it is used externally as an ointment to treat insect bites, and its oil used directly on the skin to treat acne [16]. Basil essential oil contains biologically active compounds that display insecticidal, anti-nematodes, anti-fungal and anti-bacterial effects [18].

Today, the use of beneficial soil organisms as biological fertilizers in agricultural lands is considered as most natural and desirable solution to keep the soil alive and active. The advantage of these fertilizers is mainly related to supply of organic material to the soil, due to meet its most pressing needs. In addition, the supply of nutrients perfectly consistent with natural need of plants, contributing to biodiversity, increased life activities, improve the quality and health of the environment are the main benefits of biofertilizers [25]. The use of biofertilizers has been identified as an alternative to chemical fertilization to increase soil fertility in sustainable agricultural production [30]. Many soil microorganisms are known to be able to stimulate plant growth and these have been classified as plant growth-promoting rhizobacteria (PGPR) [30]. Some of these bacteria that have ability to form a symbiosis with plants are belonging to the genus *Pseudomonas*, *Azotobacter*, *Azospirillum* and *Bacillus* sp. [9, 19]. Bacteria of the genus *Azotobacter* and *Azospirillum* are important plant growth promoting bacteria that produces significant amounts of hormones specially auxin, gibberellin and cytokinin that stimulate the growth, development and yield of plant in addition to biological nitrogen fixation [20]. In *Azospirillum*, despite their nitrogen fixing ability, the increase in yield is mainly attributed to improved root development due to the production of growth promoting substances and consequently increased rates of water and mineral uptake [9]. In anise plant, *Azotobacter* in amount of six liters per hectare with 50 plants per square meter showed the highest biomass yield [11]. Jahan et al [12] found that the application of manure and biofertilizers increased yield and quality of hulless pumpkin (*Cucurbita pepo* L.). In sage (*Salvia officinalis* L.) using biological fertilizers containing *Azospirillum* and *Azotobacter* enhanced height and dry and fresh weight of plant shoots in first and second harvest during two seasons [2]. Use of biological and chemical fertilizers of nitrogen increased plant harvest index in comparison to control and the highest amount of harvest index was recorded for combination of bio-fertilizer and 50% chemical fertilizer [13].

Arbuscular mycorrhizal fungi are major components of rhizosphere microflora in natural ecosystems [7], which has a symbiotic relationship with several species of medicinal angiosperms [5, 26]. Several reports have shown that growth and nutrient uptake was increased in plants inoculated with mycorrhizal fungi and consequently enhanced tolerance to environmental stresses and disease as well as yield [4, 17]. Khorramdell et al [29] reported that inoculating with *Azotobacter*, *Azospirillum* and mycorrhiza increased net assimilation rate and crop growth rate in *Nigella sativa* plants. In palmrosa (*Cymbopogon martinii*), combined application of mycorrhiza fungi with *Azospirillum* and *Bacillus* bacteria increased in biomass production [24]. Toussaint et al. [8] observed that the application of the two species of mycorrhiza fungus, *Glomus mosseae* and *G. caledonium*, dramatically increased plant yield and P concentration. Inoculating with the fungus *Glomus etunicatum* increased the growth and physiological parameters of basil [16].

Citric acid is a six-carbon organic acid that plays an important role in the mitochondrial citric acid cycle and produce cellular energy through oxidative phosphorylation. This compound is formed by adding acetyl coenzyme A to oxaloacetic acid [27]. Used a combination of citric acid and malic acid increased plant dry and fresh weight, postharvest quality and essential oil in comparison with control [22]. In basil, foliar spray of citric acid significantly increased the amount of essential oil where the 0.1% treatment had the highest affect [23].

Because application of biological fertilizers on medicinal plants is relatively new topic, there is a lack of information about the response of these species to biological fertilizers, and little work has been done in this field. It seems that even when the yield of these plant resulted in the use of biological fertilizers is less or equal to chemical fertilizers, production of these plants using natural inputs such as organic fertilizers, may be suitable solution for producing healthy drug. Therefore, the aim of this study was to investigate the effect of inoculation with mycorrhiza fungi, *Azotobacter* and *Azospirillum* bacteria as a biological fertilizer and foliar spray of citric acid on vegetative parameters of basil.

MATERIALS AND METHODS

The experiment was carried out in 2010-2011 at the research field of Islamic Azad University Karaj branch, Iran (35º45' N, 51º56' E, 1313 m above the sea level). Experimental design was factorial on the basis of randomized complete block design with four replications. Some of the chemical and physical properties of the experimental field soil are given in Table 1. Treatments consisted of inoculation with mycorrhiza (*Glomus intraradices*) at two levels (with and without inoculation) with a population of 250 to 300 spores per seed and inoculation with free living *Azotobacter* bacteria (*Azotobacter chroococcum*) and *Azospirillum* (*Azospirillum lipoferum*) each at two levels (with and without inoculation) with a population of 108 per mm in each of the materials used for inoculation was prepared by the Soil and Water Research Laboratory, Institute of Biology. Citric acid was sprayed at two levels (0 and 5 mM,) in three times at 10 days intervals and started at 5-6 leaf stage.

Basil seeds were planted in late May. Each plot consisted of six rows of 2.5 m length, among which plants were planted 50 cm apart with 5 cm spacing in each row. Sowing depth was about 1-2 cm. Weeds were controlled by hand weeding. Irrigation was performed every four days. Plants were harvested two months after planting. Ten plants from each plot were randomly selected for sampling. After harvesting, roots were separated from plant shoots and weighed and then placed in an oven for 48 hours at 72°C and re-weighed. Data analysis was performed by SAS software. Mean comparisons was performed using Duncan Multiple Range Test at 5% level. Excel program was used to draw graphs.

Table 1. Some of chemical and physical properties of experimental field soil.

S	Si	C	Texture	P a.v.a (mg/kg)	K a.v.a (mg/kg)	N (%)	O C (%)	pH	EC (dS/m)	Soil depth (cm)
44	22	34	C.L	7.68	240	0.17	0.85	7.81	5.82	0-30

RESULTS AND DISCUSSION

Analysis of variance showed that the effect of mycorrhiza on shoot fresh weight was not significant (Table 2). Effect of foliar spraying of citric acid on shoot fresh weight was significant at 1% level (Table 2), and shoot fresh weight following spraying with citric acid (12689.7 kg ha^{-1}) was significantly higher than those of the without citric acid (9418.8 kg ha^{-1}) (Figure 1). In dill plant, the highest shoot fresh weight was obtained by foliar spraying of citric acid in rate of 0.3% [22], which is in agreement with the results of this study. It seems that foliar spray of citric acid as an organic acid could result in stimulation or increase in proton pump activity of roots. This stimulatory effect may be also occurring with foliar spray application and transferred to root and resulting an increase in organic acids and protons efflux. This can increase the uptake of ions such as nitrogen and phosphorus by plant, which ultimately enhances yield and plants fresh weight [22]. Inoculating with *Azotobacter* and *Azospirillum* bacteria had not significant effect on shoots fresh weight (Table 2).

The test for interaction showed that the interaction of citric acid × *Azospirillum* was significant on shoot fresh weight at the 5% level (Table 2) and mean comparison with Duncan's test at the 5% level showed that the highest shoot fresh weight was obtained with combination of foliar spraying of citric acid and without Azospirillum bacteria (13691. 87 kg ha^{-1}) and the lowest obtained by combination of foliar spraying of citric acid and without Azospirillum (9287.5 kg ha^{-1}) (Figure 2).

The interaction effect of mycorrhiza × citric acid × *Azotobacter* was significant on shoot fresh weight at 5% level (Table 2), and the highest shoot fresh weight was observed in combination of foliar spraying of citric acid and inoculated with mycorrhiza and *Azotobacter* (13558.75 kg ha^{-1}) and the lowest by combination of no foliar spraying of citric acid and non-inoculated with *Azotobacter* (8625 kg ha^{-1}) (Fig. 3). Inoculating with the mycorrhiza fungus increased shoot fresh weight in green and purple variety of basil [16].

Research has shown that mycorrhiza enhanced the transport of nitrogen and phosphorus from plant or soil to rice grain and increased harvest index [21]. Synergistic effects between bacteria and fungi may aggravate the effects of fertilizer and improved crop growth and ultimately increase shoot fresh weight. In addition, as previously mentioned, the use of citric acid as an organic acid and root proton pump enhancer, has led to increase of shoot growth. In hyssop plant, combined treatment of mycorrhiza microorganisms and *Pseudomonas fluorescence*, due to synergistic effects of bacteria and fungi, led to intensify effects of fertilizer and improve plant growth and ultimately increased shoot fresh weight [1]. The study of Ratti et al [24] showed that the combined application of mycorrhiza fungi, *Azospirillum* and *Bacillus* bacteria led to increase in biomass production in palmrosa (*Cymbopogon martinii*). In the combined application of fluorescent *Pseudomonas*, mycorrhiza and / or Azotobacter diazotroph bacteria and *Azospirillum* with phosphate solubilizing bacteria and mycorrhiza fungi, because of different effects of these microorganisms on nitrogen fixation and availability of P for plants, increasing trend was observed in improved plant growth [1]. Many researchers point out the positive role of PGRP on plant growth and development, and attributed it to leakage of plant hormones, production and release of organic acids in soil, nitrogen fixation, and finally the positive interaction between them and other soil microorganisms [12].

Analysis of variance showed that the effect of inoculation with mycorrhiza, *Azotobacter* and *Azospirillum* on root fresh weight were not significant (Table 2). Effect of foliar spraying of citric acid on root fresh weight was significant at 1% level (Table 2), and the root fresh weight following spraying with citric acid (4920 kg ha^{-1}) was significantly higher than those of the without citric acid (4641.9 kg ha^{-1}) (Fig. 4).

The test for interaction showed that the interaction of citric acid × Azotobacter was significant on root fresh weight at the 1% level (Table 2) and the highest root fresh weight was obtained with combination of foliar spraying with citric acid and non-inoculated with *Azotobacter* bacteria (4987.94 kg ha^{-1}) and the lowest obtained by no foliar spraying with citric acid and non-inoculated with Azotobacter (4415.12 kg ha^{-1}) (Fig. 5).

The interaction effect of mycorrhiza × citric acid × *Azotobacter* was significant on root fresh weight at 1% level (Table 2), and the highest root fresh weight was observed in combination of foliar spraying with citric acid and inoculated with mycorrhiza and non-inoculated with *Azotobacter* (5156.87 kg ha^{-1}) and the lowest by combination of no foliar spraying with citric acid and non-inoculated with *Azotobacter* (4287 kg ha^{-1}) (Fig. 6). Inoculated with the mycorrhiza fungus was increased shoot fresh weight in green and purple variety of basil [16] which was in accordance with present study.

The interaction effect of *Azospirillum* × citric acid × *Azotobacter* was significant on root fresh weight at 1% level (Table 2), and the highest root fresh weight was observed in combination of foliar spraying with citric acid and inoculated with *Azotobacter* and *Azospirillum* (5250.75 kg ha^{-1}) and the lowest by combination of no foliar spraying with citric acid and non-inoculated with *Azotobacter* and *Azospirillum* (Fig. 7). Effect of *Bacillus*, *Azotobacter* and *Azospirillum* bacteria alone or in combination with each other on the growth and yield of *Apium graveolense* indicates that the application of these bacteria led to produce plant growth stimulating substances in rhizosphere environment and accompanied with better root growth and higher yield in comparison with non-inoculated treatment [6].

The interaction effect of *Azospirillum* × mycorrhiza × *Azotobacter* was not significant on shoot dry weight (Table 2).). Effect of foliar spraying of citric acid on shoot dry weight was significant at 1% level (Table 2), and the highest and lowest amount of shoot dry weight was obtained in spraying citric acid (3657 kg ha^{-1}) and without citric acid spraying (2908.1 kg ha^{-1}), respectively (Fig. 8). In dill plant, foliar spraying of citric acid increased shoot dry weight [22], which was in agreement with the results of this study.

A test for interaction showed statistically significant effect of mycorrhiza ×citric acid × *Azotobacter* on shoot dry weight at 5% level (Table 2) and comparisons of means showed that the highest and lowest amount of shoot dry weight was observed in non-inoculated mycorrhiza × citric acid × non-inoculated with *Azotobacter* (3939.35 kg ha^{-1}) and non-inoculated mycorrhiza × citric acid × non-inoculated Azotobacter treatments (2697.7 kg ha^{-1}) (Fig. 9). Table of data analysis showed that the effect of foliar spraying with citric acid on root dry weight was significant at 5% level (Table 2), and the root dry weight following spraying with citric acid (3263.34 kg ha^{-1}) was significantly higher than those of the without citric acid (3139.75 kg ha^{-1}) (Fig. 10). The interaction effect of citric acid × *Azotobacter* was significant on root dry weight at 1% level (Table 2), and the highest root dry weight was observed in combination of foliar spraying with citric acid without inoculated with *Azotobacter* (3286.31 kg ha^{-1}) and the lowest obtained by combination of no foliar spraying with citric acid and non-inoculated with *Azotobacter* (3228.44 kg ha^{-1}) (Fig. 11). The interaction effect of *Azospirillum* × *Azotobacter* was significant on root dry weight at 1% level (Table 2), and the highest root dry weight was observed in combination of inoculated with *Azotobacter* and *Azospirillum* (3290.81 kg ha^{-1}) and the lowest obtained by combination of non-inoculated with *Azotobacter* and inoculated with *Azospirillum* (3041.19 kg ha^{-1}) (Fig. 12).

It seems that mutual synergy effects that resulted from the combined use of these bacteria may be led to increase the root dry weight in *Azotobacter* × *Azospirillum* treatment. The main advantages of bacteria are production of plant growth stimulating and regulating hormones, development of root systems and improved water absorption and nutrition [10].

Table 2. Analysis of variance for studied traits in basil plant

SOV	df	Mean Square (MS)			
		Shoot fresh weight	root fresh weight	Shoot dry weight	Root dry weight
Block	3	15714805*	1290438 *	1329981*	337536*
Mycorrhiza (A)	1	443889 ns	11908 ns	205 ns	38171 ns
Citric acid (B)	1	171184514*	1236822 *	8973319*	244406*
Azotobacter (C)	1	467514 ns	403701 ns	13959 ns	124873 ns
Azospirillum (D)	1	12136514 ns	98517 ns	169455 ns	80869 ns
A × B	1	1732514 ns	606 ns	134799 ns	8349 ns
A × C	1	2201514 ns	8122 ns	176610 ns	44785 ns
A × D	1	1914764 ns	55283 ns	81653 ns	55991 ns
B × C	1	3482889 ns	1390335*	115702 ns	288503*
B × D	1	20554889*	108487 ns	556739 ns	55755 ns
C × D	1	2453139 ns	599269 ns	106961 ns	416186*
A × B × C	1	21471639*	1439100*	1597064 *	247879*
A × B × D	1	217389 ns	605867 ns	594 ns	101044 ns
A× C × D	1	5599139 ns	1947 ns	235370 ns	534 ns
B× C × D	1	2299014 ns	3142199 *	319394 ns	1552827*
A × B×C × D	1	46764 ns	151612 ns	319394 ns	77/49784 ns
Error	45	3998983	165012	37075	35282
CV (%)	-	18.09	8.49	14.62	5.87

*ns, nonsignificant; **, significant at P≤0.01; *, significant at P≤0.05.*

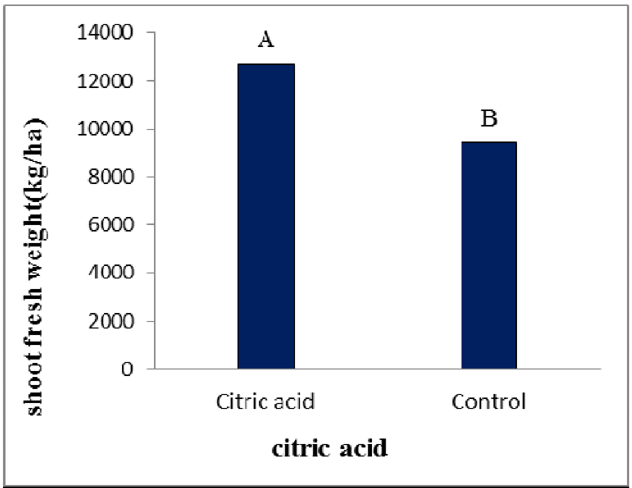

Fig. 1. Effect of citric acid on shoot fresh weight.

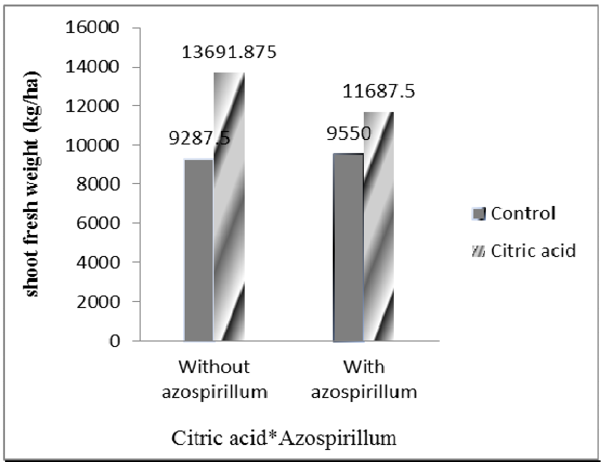

Fig. 2. Effect of citric acid and *Azospirillum* on shoot fresh weight.

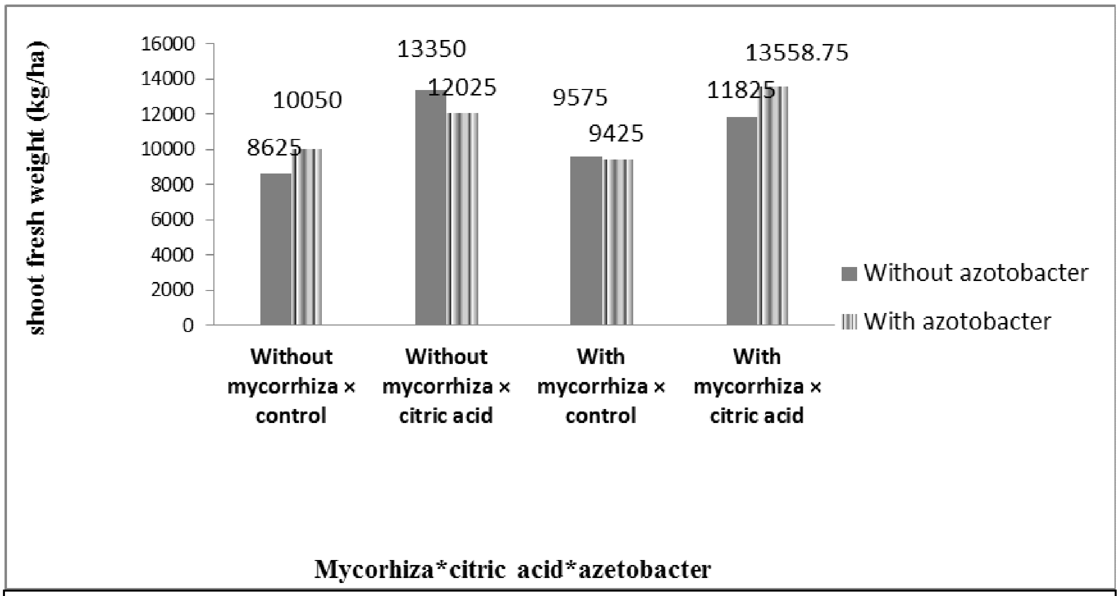

Fig. 3. Effect of mycorrhiza, citric acid and *Azotobacter* on shoot fresh weight.

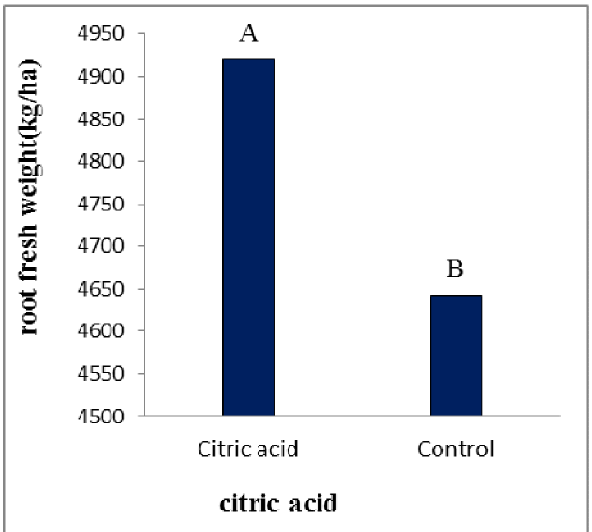

Fig. 4. Effect of citric acid on root fresh weight.

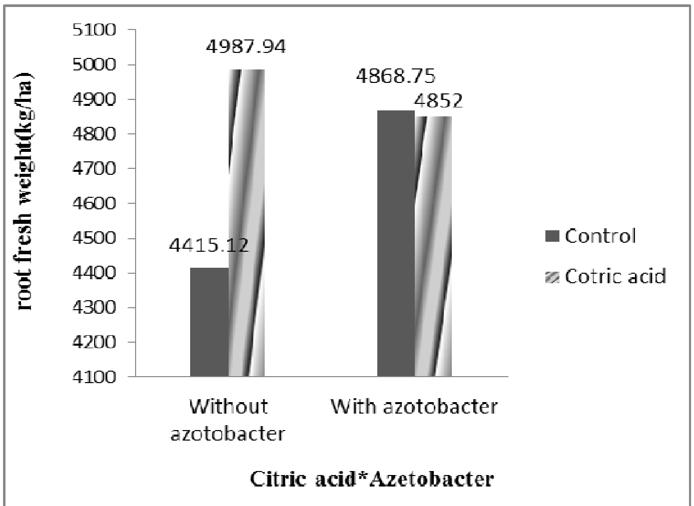

Fig. 5. Effect of citric acid and *Azotobacter* on root fresh weight.

A test for interaction showed statistically significant effect of mycorrhiza × citric acid × *Azotobacter* on root dry weight at 5% level (Table 2) and comparisons of means showed that the highest and lowest amount of shoot dry weight was observed in inoculated mycorrhiza × without citric acid spray × inoculated with *Azotobacter* (3326.75 kg ha^{-1}) and inoculated mycorrhiza × without citric acid spray × non-inoculated *Azotobacter* treatments (2926.7 kg ha^{-1}) (Fig. 13). Synergistic effects between bacteria and fungi may aggravate the effects of fertilizer and improved plant root development. The interaction effect of *Azospirillum* × citric acid × *Azotobacter* was significant on root dry weight at 5% level (Table 2), and the highest root fresh weight was observed in combination of foliar spraying with citric acid and non inoculated with *Azotobacter* and *Azospirillum* (3528.75 kg ha^{-1}) and the lowest by combination of foliar spraying with citric acid and inoculated with *Azotobacter* and non inoculated *Azospirillum* (3010 kg ha^{-1}) (Fig. 14).

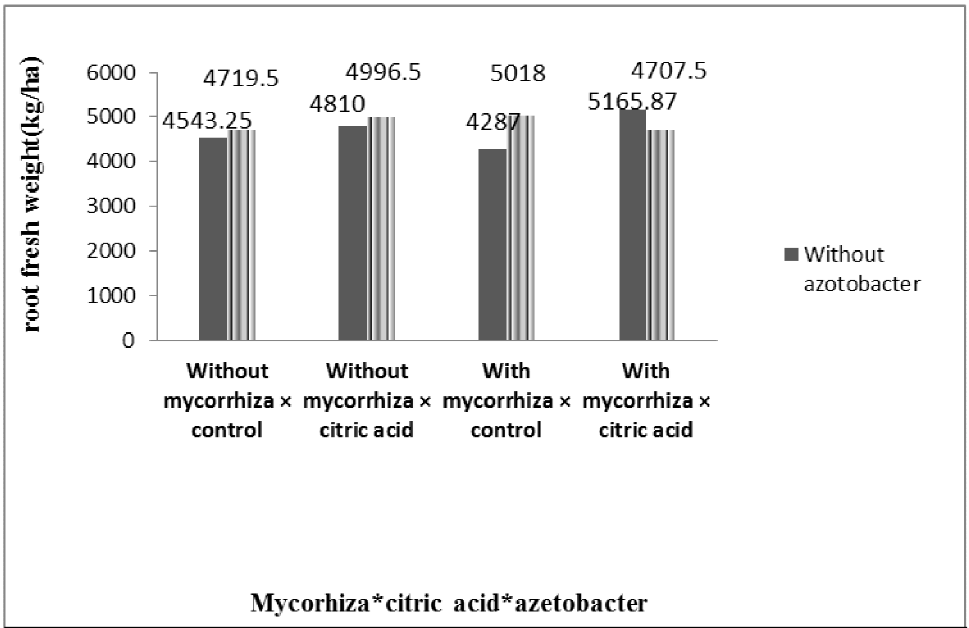

Fig. 6. Effect of mycorrhiza, citric acid and *Azotobacter* on root fresh weight

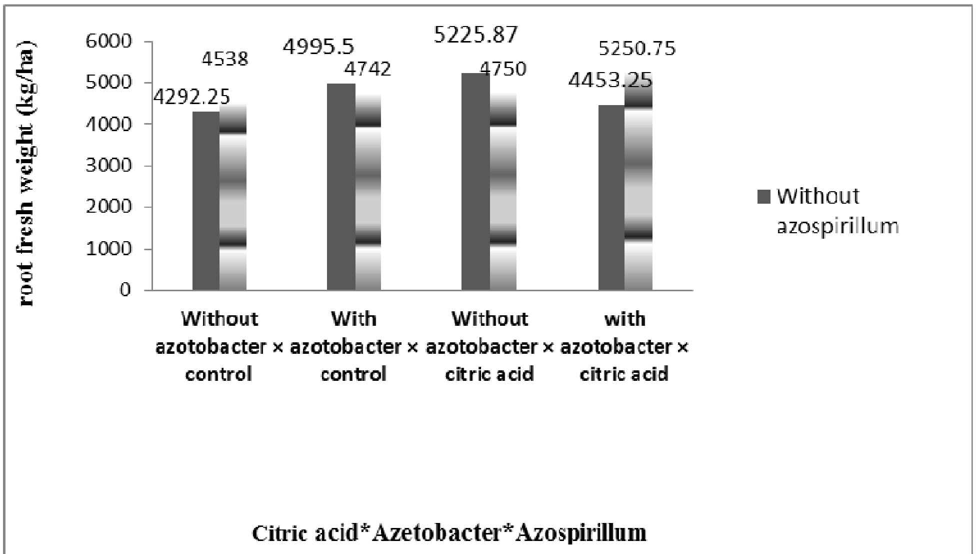

Fig. 7. Effect of citric acid, *Azotobacter* and *Azospirillum* on root fresh weight.

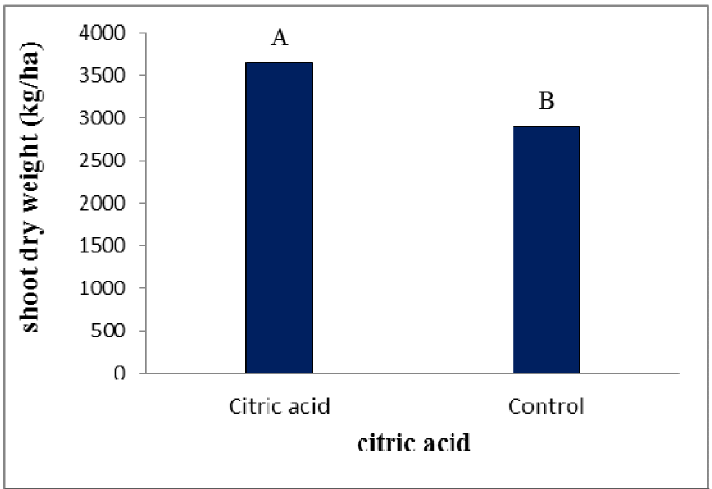

Fig. 8. Effect of citric acid on shoot dry weight.

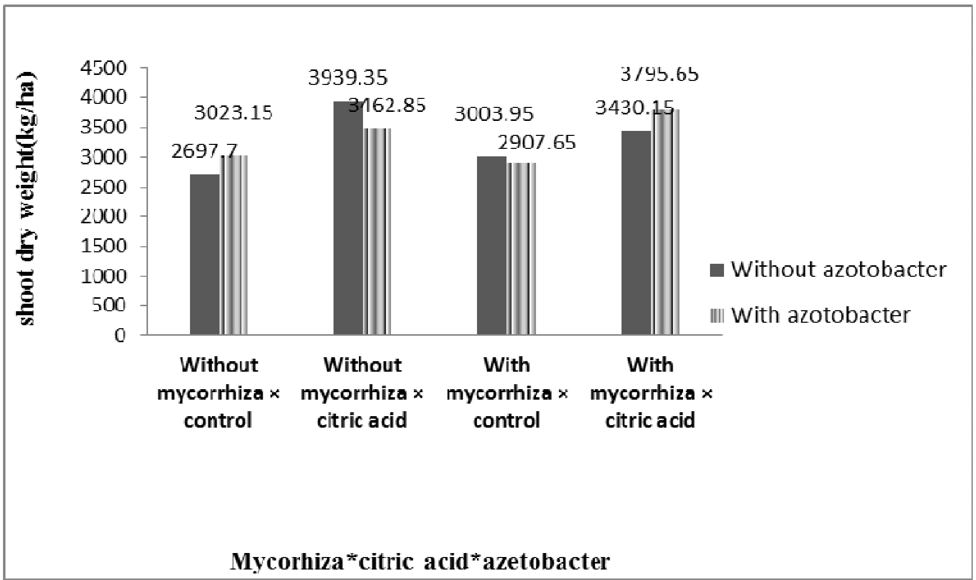

Fig. 9. Effect of mycorrhiza, citric acid and *Azotobacter* on shoot dry weight.

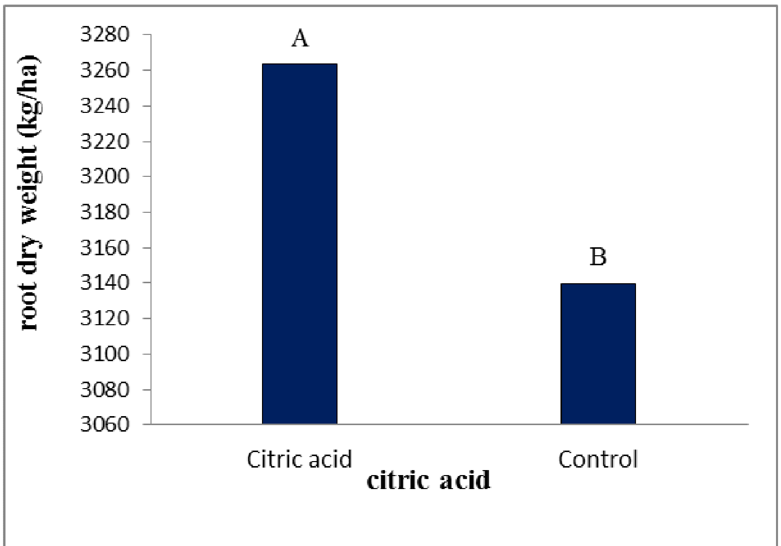

Fig. 10. Effect of citric acid on root dry weight.

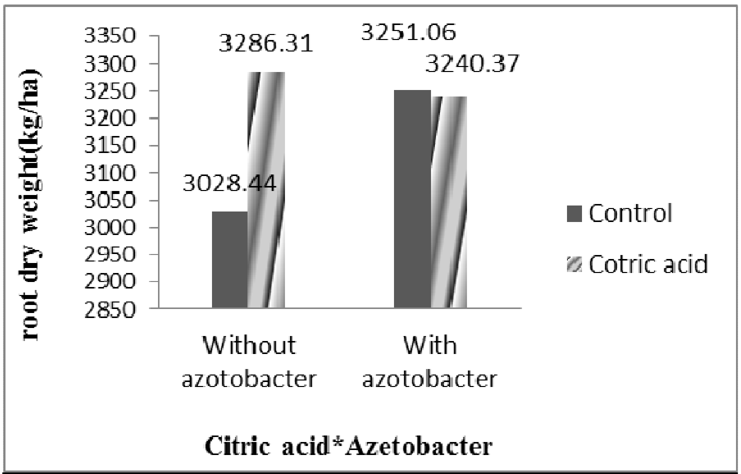

Fig. 11. Effect of citric acid and *Azotobacter* on root dry weight.

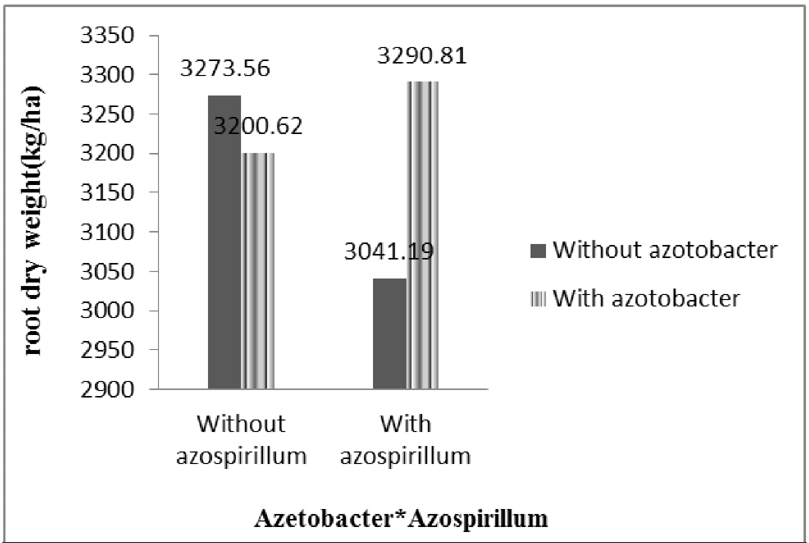

Fig. 12. Effect of *Azotobacter* and *Azospirillum* on root dry weight.

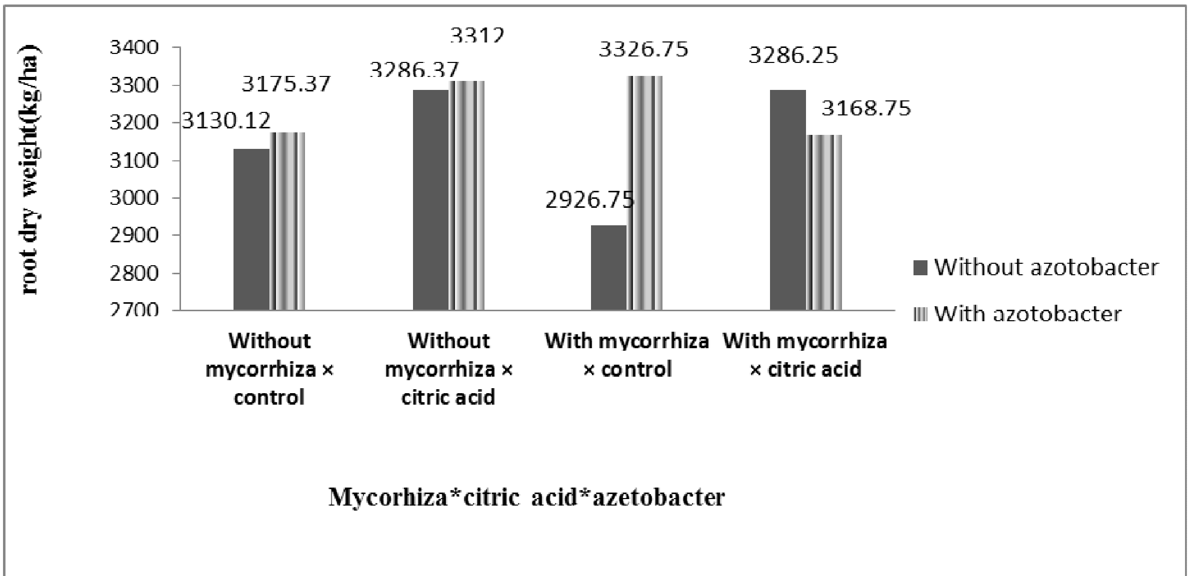

Fig. 13. Effect of mycorrhiza, citric acid and *Azotobacter* on root dry weight.

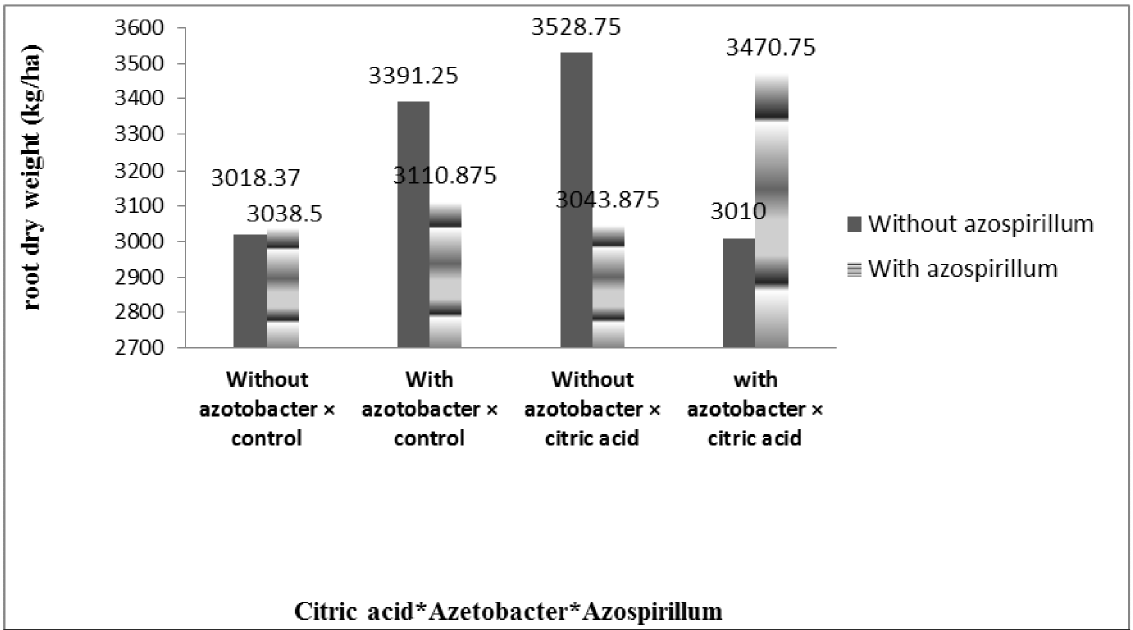

Fig. 14. Effect of citric acid, *Azospirillum* and *Azotobacter* on root dry weigh

CONCLUSION

Considering the current global effort to eliminate the use of synthetic and chemicals input in food production and natural and organic agricultural production is of high importance, the results of this study show that the use of citric acid as a foliar spray of organic compound instead of using nitrogen fertilizers, can help to improve plant growth and yield as well as production of safe product. In addition, it can also be achieved by combined use of biological fertilizers.

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