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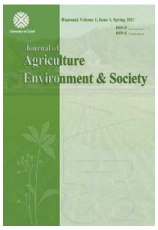

Changes in morpho-physiological characteristics of peppermint by foliar application of biofertilizer and nanofertilizers

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ABSTRACT

Peppermint is one of the medicinal and aromatic plants that is widely used as a pharmaceutical, food, and cosmetic. In recent years, due to the harmful effects of fertilizers on the environment, their use has been criticized. Using bio and nano-fertilizer as an alternative source for conventional fertilizers leads to the gradual realization of fertilizer nutrients in the soil and in a controlled manner. The use of these fertilizers leads to increased nutrients, use efficiency, reduce the toxicity of the soil, and reduce the frequency of the application. The current study evaluates the effect of nano- fertilizers and organic fertilizers on morpho-physiological characteristics of peppermint based a completely randomized design. Experimental treatments were the foliar application of nano fertilizers (nitrogen, iron, and potassium) and normal vermiwash, enriched vermiwash, tea compost, and control. The results showed that the effect of nano- fertilizers and bio-fertilizers on plant height, leaf area, leaf dry weight, total dry weight, photosynthetic pigments, proline, protein, and carbohydrate were significant. All treatments increased photosynthetic pigments, compared to control. The maximum amount of proline, total dry weight, and plant height was obtained using nano fertilizers of nitrogen whereas enriched vermiwash resulted in the highest protein and leaf area.

1. Introduction

Mint is among those medicinal plants, which, due to medicinal effects and alimentary use, have been of interest to researchers since a long time ago. It has been used as spice and medicine since two thousand years ago. However, the use of peppermints (*Mentha piperita* L.) by humans started only 250 years ago. Leaves, vegetative parts, and essential oil of this plant have been cited as medicines in the most reliable pharmacopeias. Active ingredients of peppermints are used in food, cosmetics, pastry, soft drink, and spice industries (Omidbeige, 2006). This plant is among those valuable medicinal plants, the effects of which have been shown for the prevention and treatment of irritable bowel syndrome, based on recent studies (Izadi et al., 2009). Peppermints has medicinal properties and many applications, such as anti-gas and antibacterial medicines and is used as a constituent of medicinal plants produced in Iran, such as Altadine, Masoument, mint drop, Menta, and Alicom (Omidbeigi, 2006).

Nanotechnology is a science and technology which has recently attracted much attention. This technology, which is an approach in every field of study, has the ability to produce modern materials, tools, and systems by manipulation of atomic and molecular levels (Lane, 2001). One of the most important applications of nanotechnology in different agricultural fields in the water and soil part, is the use of nano fertilizers for plant nutrition. Nowadays, biofertilizers are considered an alternative for chemical fertilizers, with the goal of increasing soil fertilization and producing yields in sustainable agriculture (Wu et al., 2005). Biofertilizers contain living cells of different microorganism types, which prepare the conditions for plants to absorb nutritive elements through biological processes (Han et al., 2006). Usually, these microorganisms cause the production of compositions such

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as gibberellin, cytokinin, and auxin, facilitation of the absorption of water and nutritive elements, especially phosphorus, nitrogen, and microelements from the soil, and disease reduction or prevention in plants (Hayat, 2010). The difference between biofertilizers and organic and chemical fertilizers is that they don't directly provide any nutritive elements for the plant (Han et al., 2006).

Vermiwash is a liquid containing plant growth stimulants, collected after the water has moved through a column of active worms (the worms used in the production of vermicompost) and can be used as a foliar application on plant leaves. Enriched vermiwash is obtained by a mixture of normal vermiwash enriched with aquarium water and bioreactor with extracts from Nettles and *aloe vera* plants, and its application, in addition to having positive effects on economic aspects and better performance, helps bioenvironmental sustainability. The extract obtained by the solution of water and vermicompost is called compost tea and is considered one of the best inputs in organic agriculture.

The results of one study have shown that the essential oil percentage of the medicinal plant peppermint for the Azotobacter and Azospirillum treatment is equal to those of the treatments with chemical fertilizer application (Kalra, 2003). In a study by Mehrafarin et al (2011), the results indicated that the fertilizer treatments had a significant effect on the performance of medicinal compositions of the plants, including the amounts of the essential oil, menthone, menthol, and biofertilizers significantly increased the amounts of the essential oil, menthone and menthol compared to the control treatment. Abdu and Mohammad (2014) reported that the consumption of 46 t/ha of compost increases the performance of the dry weight and the wet weight by 43 percent, compared to common complete fertilizers. In a study, the possibility of using biofertilizers instead of chemical fertilizers was investigated for the plant fennel, and the results were indicative of an increase in the vegetative growth, the performance, and the amounts of essential oil for biofertilizer treatments (Azzaz et al., 2009). In another study, the results of biofertilizer consumption by Ajwain (*Carum copticum*) showed the significant effect of biofertilizers on all of the characteristics. The highest biological performance, seed performance, essential oil performance, and content were obtained by the treatment seed inoculation + foliar application in the stem elongation stage (Ghilavizadeh et al., 2013).

Since the global approach in the production of medicinal plants is towards the improvement of quantity and quality of active ingredients, the application of nano fertilizers and biofertilizers is examined in this study.

2. Materials and Methods

This study was performed as a pot experiment. For this purpose, firstly the peppermint rhizomes were divided into parts with 3 nods and transported to plastic pots with crater diameters of 20 cm and lengths of 18 cm, the soils of which contained farm soil and sand and vermicompost with the ratio 1:1:2. The experiment was performed as a completely randomized design with 7 treatments and 3 replications. The plants were regularly watered with normal water. 3 weeks after the plants had reached the appropriate growth, the stems were headed back from a height of 5 cm. Then, the fertilizer treatments, including nitrogen nanofertilizer, iron nanofertilizer, potassium nanofertilizer (with the concentration of 2%) and normal vermiwash, enriched vermiwash, compost tea (with the concentration 1 in 3 water) and the control, were applied as foliar applicationin 4 repetitions during a course of 8 weeks.

2.1. Characteristics under investigation:

Measurement of the photosynthetic pigments: To do this, the Arnon (1949) method was used. For this purpose, 0.1 of the fresh weight of the leaf was groundusing 10 ml of 80 percent acetone in aporcelain mortarand, then, the solution was moved through a filter paper. The filtered solution became ready for examination by a spectrophotometry device of JENUS UV-1200 model. After the calibration of the device, each sample was examined for three wavelengths of, 663 nanometers for the chlorophyll a examination, 645 nanometer for the chlorophyll b examination and 470 nanometer for the examination of carotenoids and xanthophyll. The numbers relating to the absorptions were put to the following formulae and the amounts of the chlorophyll a and chlorophyll b and the sum of carotenoid and xanthophyll were calculated using the suggested equations by Arnon (1949).

The measurement of amino acids in leaf proline was performed by using Bates et al (1973) method. For this, 0.5 grams of the sample wasground using liquid nitrogen in aporcelain mortar. Then, 10 ml of 3 percent sulfo saliscylic acid was added to the sample and it was ground again. The resulting solution was placed for 15 minutes in a 10000 revolutions per minute centrifuge. 2 ml of the liquid was taken from the surface of the centrifuged solution and, under the hood, 2ml acetic acid and 2ml of ninhydrin reagent (1.25 gram ninhydrin + 30 milliliters of glacial acetic acid + 20 milliliters of 6 Molar phosphoric acid) were added to it and it was mixed for 15 minutes at 40 degrees centigrade by the mixer. The solution obtained was placed in a bain-marieof 100 degrees centigrade for 1 hour. Then, it was placed in a cold water bath for 10 minutes and, then, 4 ml of toluene was added to it. The tube contents were intensely mixed to obtain two phases. In order to read the absorption amount in the spectrophotometry device, toluene was used as the control, and in order to measure the proline amount, the absorption amount of the top phase was measured in the wavelength of 520 nanometers. In order to measure the protein concentrations, one gram of plant tissue and one milliliter of 50 milli molartris-hcl extraction buffer with a pH of 7 containing 10 mM $MgSO_4$, 2 mM EDTA, 20 mM DTT, 10% glycerol (V/V) and 2% PVP (V/W) were ground in the mortar and, then, the solution was transferred to the centrifuge tube and centrifuged at 4 degrees centigrade for 20 minutes in 13000 g by a refrigerated centrifuge machine. Then, the floating solution was collected and, once again, centrifuged for 20 minutes in 13000 g and, then, transferred to an eppendorf. The volume of the resulting supernatant was recorded and it was placed for short term preservation at 70 degrees centigrade.

The resulting protein extractswere used to measure the protein. In order to measure the quantity of the protein existing in the extracted protein extracts, the Bradford (1976) was used. To measure the soluble sugar (Kochert, 1978) 0.1 grams of dried leaves plus 10 milliliters of 70% ethanol were placed in a refrigerator for one week, so that its soluble sugars dissolve. Then, samples of 0.5 ml were taken from the above mentioned solution and their volumes were increased to 2 ml by the addition of distilled water, and 1 ml of 5% phenol was added to them and they were mixed by a vortex. Thereafter, 5 ml of concentrated sulfuric acid was added to every one of them under pressure. After half an hour, the wavelength 485 was used by the spectrophotometer device to measure glucose. The sugar quantities of the solutions were evaluated using the standard curve based on mg. kg DW^{-1}. Also, in this experiment, the characteristics leaf area (by leaf area meter device), plant height, dry leaf weight and the number of lateral branches were evaluated.

Table 1. Mean comparison for investigating the effect of different treatment proline and photosynthetic pigments of Peppermint

Treatment	Proline (μM/g)	Carotenoids (mg/g FW)	Chlorophyll b (mg/g FW)	Chlorophyll a (mg/g FW)
Nano fertilizers of nitrogen	0.25 b	0.222 b	1.09 b	2.02 b
Nano fertilizers of potassium	0.054 d	0.196 d	0.877 e	1.82 e
Nano fertilizers of iron	0.104 d	0.197 d	0.91 d	1.86 d
Tea compost	0.814 d	0.194 d	0.878 e	1.8 f
Normal vermiwash	0.125 c	0.207 c	0.94 c	1.87 c
Enriched vermiwash	0.145 a	0.229 a	1.12 a	2.04 a
Control	0.158 e	0.180 e	0.78 f	1.7 g

Means followed by the same letter(s) are not significantly different at 0.05 level of probability

2.2. Statistical analysis of the data

The statistical analysis of the data was performed by using the SPSS software and the mean comparison of the data was done by using the Duncan test.

3. Results

3.1. Effects of nanofertilizers and biofertilizers on photosynthetic pigments

Based on the results, the experimental treatments had significant effects on the amounts of the chlorophylls a and b at 5% level. All of the experimental treatments caused increases in the amounts of the chlorophylls a and b and the highest increase was observed for the enriched vermiwash treatment, with which the amount of the chlorophyll a has increased by 20 percent compared to the control, and the amount of the chlorophyll b has increased by 43 percent. Following the enriched vermiwash, nitrogen nanofertilizer had the greatest effect, with an18 percent increase of the chlorophyll a and a 39 percent increase of the chlorophyll b (Table 1).

Based on the results, the experimental treatments were significant on the carotenoids amount at 5% level. The greatest effect compared to the control was related to the experimental treatment enriched vermiwash, which had a 37 percent increase compared to the control, and the other experimental treatments had less increasing effects than the control (Table 1).

3.2. Effects of various nanofertilizers and biofertilizers on leaf proline content

Experimental treatments had significant effects on the synthesis amount and proline accumulation in the peppermint plant at 5% level. Except for the experimental treatment nitrogen nanofertilizer, which had the highest positive effect and caused a 60 percent increase in proline compared to the control and its difference from the control was significant, all of the other experimental treatments had negative and decreasing effects compared to the control, and the least negative effect was related to the experimental treatment potassium fertilizer, with a 66 percent decrease in proline compared to the control (Table 1).

3.3. Effects of various nanofertilizers and biofertilizers on leaf soluble sugars

The results showed that, the effects of experimental treatments on the amounts of carbohydrates, which were reductases of peppermint leaves were significant at 5% level. The experimental treatment normal vermiwash had the greatest positive effect on the amounts of reductive carbohydrates and caused a 23 percent increase compared to the control, and the least positive effect was for the experimental treatment enriched vermiwash, and the other experimental treatments had negative effects compared to the control. The experimental treatment nitrogen nanofertilizer had the greatest negative effect and caused a 44 percent decrease inreductive carbohydrates compared to the control (Figure 1).

3.3. Effects of various nanofertilizers and biofertilizers on leaf soluble sugars

The results showed that, the effects of experimental treatments on the amounts of carbohydrates, which were reductases of peppermint leaves were significant at 5% level. The experimental treatment normal vermiwash had the greatest positive effect on the amounts of reductive carbohydrates and caused a 23 percent increase compared to the control, and the least positive effect was for the experimental treatment enriched vermiwash, and the other experimental treatments had negative effects compared to the control. The experimental treatment nitrogen nanofertilizer had the greatest negative effect and caused a 44 percent decrease inreductive carbohydrates compared to the control (Figure 1).

3.4. Effects of various nanofertilizers and biofertilizers on the leaf protein content

Experimental treatments had significant effectsat 5% level on the synthesis amount and protein accumulation in the peppermint plant leaves. The experimental treatment enriched vermiwash had higher protein amount compared to the control and caused a 7 percent increase. There was no significant difference between the experimental treatment nitrogen nanofertilizer and the control and the other treatments had decreasing effects compared to the control and the lowest negative effects were obtained for the fertilizing treatments potassium nanofertilizer and compost tea with, respectively, 15 and 17 percent decrease compared to the control, and there was no significant difference between these two treatments (Figure 2).

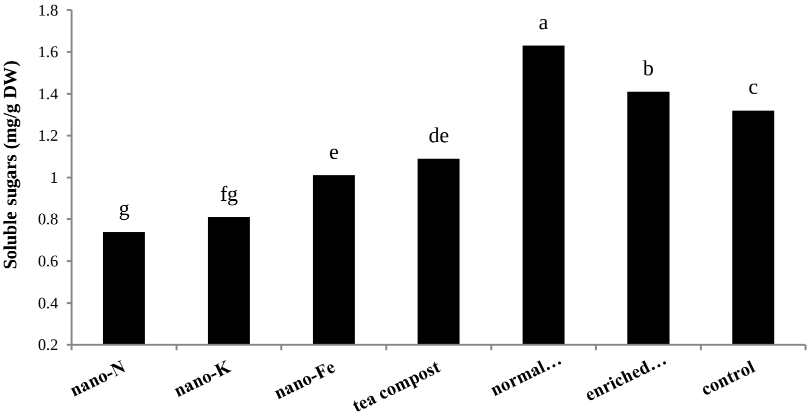

Figure 1. Effects of experimental treatments on leaf soluble sugars

3.5. Effects of various nanofertilizers and biofertilizers on the leaf area

Based on the results obtained from the variance analysis, the experimental treatments had significant effects at 5% level on the peppermint leaf area.The leaf areas of the experimental treatments enriched vermiwash and nitrogen nanofertilizer were higher than the control, with respectively 26 and 16 percent increase in the leaf areas compared to the control, and the other treatments had less leaf areas compared to the control and the least leaf area was for the compost tea treatment, with a 23 percent decrease compared to the control (Table 2).

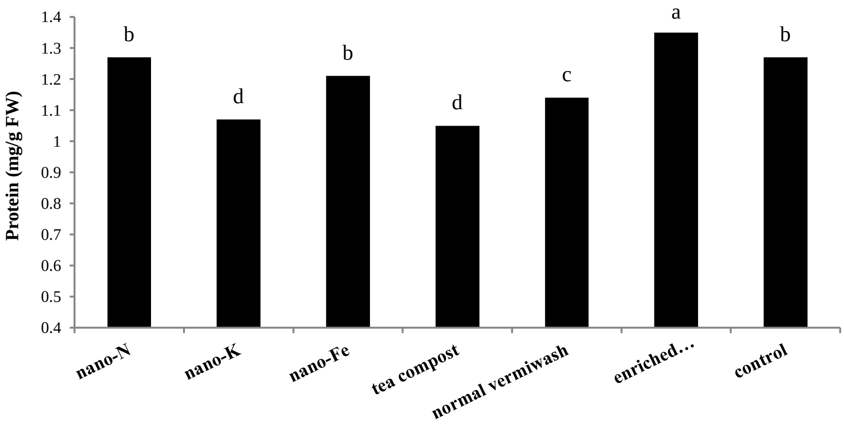

Figure 2. Effects of experimental treatments on leaf protein content

3.6. Effects of various nanofertilizers and biofertilizers on plant height

The results of variance analysis showed that the effects of various treatments on the peppermint stem length weresignificant at 5% level. All of the experimental treatments had increasing effects on the plant height compared to the control, and the highest plant height compared to the control was for the nitrogen nanofertilizer treatment with 139 percent increase, and the normal vermiwash treatment didn't have a significant difference with the control (Table 2).

3.7. Effects of various nanofertilizers and biofertilizers on total dry weight

Based on the results obtained from the variance analysis, the experimental treatments had significant effects at 5% level on the peppermint total dry weight. Except for the experimental treatment compost tea, all of the treatments had increasing effects compared to the control, and the highest amount of total dry weight was for nitrogen nanofertilizer, with 190 percent increase, and the least total dry weight compared to the control was for the compost tea treatment, with a 7 percent decrease (Table 2).

3.8. Effects of various nanofertilizers and biofertilizers on the number of lateral branches

Based on the results obtained from the variance analysis, the experimental treatments had significant effects at 5% level on the number of lateral stemsin the peppermint. All of the experimental treatments had positive effects compared to the control, and the highest number of lateral stemscompared to the control was for nitrogen nanofertilizer, with 146 percent increase, and the least positive effect was for the compost tea treatment, with a 10 percent increasecompared to the control (Table 2).

Table 2. Mean comparison for investigating the effect of different treatment on some morphological traits of Peppermint

Treatment	Plant height (cm)	Leaf area (mm^2)	Number of lateral branches	Dry weight (g)
Nano fertilizers of nitrogen	28.8 a	7846.2 a	60.7 a	4.59 a
Nano fertilizers of potassium	12.67 c	6521.6 b	29.7 cd	2.01 c
Nano fertilizers of iron	12.6 c	5385.8 c	37.0 bc	3.37 b
Tea compost	12.8 c	5157.4 c	27.3 cd	1.82 c
Normal vermiwash	12.0 c	6147.7 b	33.3 bcd	2.31 c
Enriched vermiwash	20.4 b	8545.3 a	40.7 b	3.40 b
Control	12.06 c	6736.6 b	24.6 d	1.73 c

Means followed by the same letter(s) are not significantly different at 0.05 level of probability

4. Discussion

Overall, the results of this experiment showed that the application of concentrated vermiwash and nitrogen nanofertilizer has been beneficial for most of the characteristics under study and has improved them. The chlorophyll, the carotenoids and the leaf area were among these characteristics. The chlorophyll amount in living plants is one of the important factors for preservation of the photosynthesis capacity (Jiang and Huang, 2001). One study showed that the application of high rates of vermicompost resulted in increase in the leaf area, receiving more light and increase in photosynthesis (Sallaku *et al.*, 2009). Besides nitrogen, which causes an increase in chlorophyll amount and photosynthesis in plants, vermicomposts, as well, cause an increase in chlorophyll amount and photosynthesis in plants, because of having micronutritive elements, and especially iron. According to the report by Sairam et al (1998), carotenoids, by using the xanthophyll cycle and with epoxidation and de-epoxidation reactions, reduce the oxygen consumption and protect chlorophylls against photooxydation (Ansari 2008). Increase in leaf area determines photosynthesis capacity of plants. Change in leaf area, which is affected by genotypes, plant density, weather and soil fertility, will also affect the performance (Nezarat and Gholami, 2009). Yasari and Patwardhan (2007) stated that the amount of increase in the leaf area determines the photosynthesis capacity of the plant. These researchers emphasize the significant increase in leaf area measure with simultaneous applications of nitrogen fertilizers and biofertilizers. Using biological nanofertilizers has improved the growth of this plant. Better growth and higher performance of plants are attributed to slow release of nutritive elements with gibberellin and auxindue to application of biofertilizers like vermiwash. Soluble sugars act as osmoregulators, cell membrane stabilizers and agents of turgor pressure maintenance (Slama et al., 2007). Hormones such as cytokinin, auxin, amino acids, organic acids, vitamins, enzymes such asprotease, amylase, urease, secretions and mucoid materials from earthworm body and heterotrophic bacteria, fungi, actinomycetes, nitrogen fixing bacteria (Azotobacter, Rhizobium, Agrobacterium) and some phosphate solubilizing bacteria are also present in vermiwash (Shivsubramanian and Ganeshkumar, 2004). Asgari et al (2012) reported in a study that the growth stimulant bacteria treatment had the highest plant growth among the experimental treatments (growth stimulant bacteria, vermicompost fertilizer and humic acid) for peppermint. Therefore, applications of bacteria, vermicompost and humic acid have resulted in a significant difference through the creation of better nutritive conditions for vegetative growth and the production of higher plants.

In the study performed by EI-Gendy (2006) on basil, it was observed that the effect of nitrogen on the plant growth was significant and caused an increase in height compared to the control. Asgari et al (2012) reported that the experimental treatments growth stimulant bacteria, vermicompost and humic acid had significant effects on the dry weight of peppermint leaf and the growth stimulant bacteria treatment had the highest leaf dry weight. The plant dry weight is considered as an important indication of plant growth condition. Increase in the plant dry weight was indicative of more success in photosynthesis of the plant due to the availability of a more appropriate growth condition. The research showed that, through increasing the leaf area and providing the appropriate conditions for energy absorption and, also, participating in the structures of the enzymes involved in the photosynthetic carbon metabolism, the fertilizer causes an increase in photosynthesis efficiency and the essential oil performance in peppermint (Arabci and Bayram, 2004). Fracis et al (2000), as well, showed that, with an increase in the nitrogen amount, the amount of the dry matter produced by chamomile increased due to volume increase of the plant canopy, the leaf area increase and more light absorption.

Many nanomaterials have a higher toxicity than ordinary materials. Nanomaterials are highly reactive, and the studies showed that they can result in oxidation causing harm to the cells, and they sometimes cause the deaths of cells and laboratory animals. Overall, the nanoparticle effects on living systems has not been examined sufficiently (Wang *et al.*, 2006). Whereas, sustainable agriculture based on biofertilizer consumption, with the aim of eliminating or markedly decreasing chemical input consumption, is considered a desirable solution for these problems. Biofertilizers contain conservatives with a dense population of one or more types of helpful organisms living in soil or in the form of their metabolic products, which are used to improve soil fertility and appropriately provide nutritive elements required by a sustainable agriculture system, and, taking into account the positive effect of vermiwash on peppermint characteristics under study, can be used as an alternative for chemical fertilizers.

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