Effect of Cyanobacterial Extract on Medicinal Plant *Mentha piperita* L.

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Abstract

As the simplest photosynthetic organisms, cyanobacteria are the interface between bacteria and plants in terms of evolutionary process. The positive effect of these photosynthetic microorganisms on plant growth is one of the important roles in biotechnology. Their production of various secondary metabolites is another known application of these microalgae in agricultural biotechnology. In this study, the effect of two species of heterocystous cyanobacteria, isolated from paddy fields of Golestan Province in Iran, on the growth of peppermint (*Mentha piperita* L.) was evaluated. A significant difference was observed between treated plants and control in terms of growth parameters. In addition, as one of the factors influencing the vegetative parameters of plants, the qualitative identification of phytohormones in cyanobacterial biomass was investigated by biochemical methods and High Performance Liquid Chromatography technique. The results indicated the presence of plant growth hormones such as indole acetic acid and indole butyric acid in extracts of studied cyanobacteria.

Keywords: Cyanobacteria, High Performance Liquid Chromatography, Phytohormones, Peppermint, Vegetative Parameters.

Introduction

Cyanobacteria are the simplest photosynthetic organisms (Schopf, 2000). Most cyanobacteria are aerobic photoautotroph organisms that require water, carbon dioxide, minerals, and light during their life cycles. Over recent decade, researchers have focused mostly on the biotechnology aspects of this group of photosynthetic organisms (Bhadury et al., 2004; Dahms et al., 2006). The applications of cyanobacteria in various biotechnology fields have been reported, including the production of food, fuel, bio-fertilizer, color, and various metabolites such as toxins, vitamins, enzymes and pharmaceuticals (Abed et al., 2009). There are several reports on the production of secondary metabolites by most cyanobacteria (Gademann, and Portmann 2008; Rastogi and Sinha, 2009). Many researchers classify the phytohormones synthesized by cyanobacteria as secondary metabolites. In general, the production of phytohormones is one of the unique characteristics of plants, which is also found in some cyanobacteria.

Recent studies have shown that cyanobac-

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teria can be useful for plants through the production of the plant growth regulators (PGRs) such as auxin, cytokinin, gibberellin, ethylene, jasmonic acid and abscisic acid hormones (Rodgers et al., 1979). In vivo experiments on some plants treated with these microorganisms also clearly show the production of PGRs by cyanobacteria (Metting, 1988).

Medicinal plants are useful alternatives for chemical drugs. These plants can be used to reduce many adverse effects of synthetic chemical drugs. As a medicinal plant, peppermint with a scientific name of *Mentha piperita* L. is a hybrid of *Mentha spicata* L. and *Mentha aquatica* L.. This medicinal plant is a herbaceous perennial plant which belongs to a taxonomically Lamiaceae (Lamiales, Rosidae). The essential oil of this plant has antioxidant, anti-fungal and insecticidal properties which are used in medicinal applications (Gradiner, 2000).

Due to the importance of medicinal herbs in protecting human health and considering the high value of peppermint as a medicinal herb, the effect of cyanobacteria on peppermint (*Mentha piperita* L.) was investigated. Some purposes such as the evaluation of the effect of cyanobacteria on medicinal plants growth were followed in this study.

Materials and Methods

Sampling and purification

Soil samples were collected from the farms of Hossein Abad village, 20 km from Gorgan city, Golestan Province (36° 55′ 50″ N; 54° 51′ 41″ E). 10 g of the collected soil was grounded in a porcelain mortar and moved to sterile Petri dishes containing culture media, nitrate-free BG-11 medium (pH of 7.1). Plates were placed in a culture chamber to form the algae colony for 14 days. Then, the colonies were purified in a solid culture medium (Stanier et al., 1971).

Algae culture

Two purified cyanobacteria species including *Hapalosiphon welwitschii* West & G.S.West and *Anabaena vaginicola* F.E. Fritsch & Rich were used for morphological and molecular identification. In this study, soil extract was used as cyanobacterial culture medium to obtain an algal inoculum in laboratory scale. In order to prepare the soil extracts, a suspension of 500g soil in water was prepared and extracts of mineral content were isolated. The extract was autoclaved at 121°C and pressure of 15p before inoculation of algae for 20 minutes.

The 50 ml of a three-week-old cyanobacterial stock was added to 1.5 liters of soil extract culture. It was kept under a condition of 25 ± 2°C and a light intensity of 4000 Lux (with a light period of 14 hours of light and 10 hours of darkness). The aeration was carried out manually during the culture period.

The cyanobacterial inoculum was adjusted based on 21-day culture density and concentration measurement. So that after three weeks, when the production of secondary metabolites and the cell exponential phase was recognized, the optical density of cyanobacterial culture was measured with spectrophotometer at a wavelength of 750 nm. Under culture optical concentration of 0.3

(OD = 0.3), algal suspension was homogenized with a blender.

Pot culture and inoculation of cyanobacterial extract

Pot culture was done in a greenhouse under the natural light and 20 to 25°C temperatures. peppermint (*Mentha pipertia* L.) rhizomes were prepared from the Research Institute of Medicinal Plants of Shahid Beheshti University. Then, they planted in pottery pots (with 15×15 diameter). For each pot, 1.5 Kg of soil was used (Soil contains 25% sand, 60% peat and 15% ordinary soil). In each pot, four peppermint rhizomes were planted. The pots were divided into three groups, first group was control pots which irrigated only with water; the second group was inoculated with cyanobacterial suspension, *Anabaena vaginicola*, and the third group was the plants which treated by cyanobacterial suspension, *Hapalosiphon welwitschii*. Six replicates were considered for each treatment. After two weeks planting rhizomes, the treated plants were irrigated with 200ml of cyanobacterial suspensions. The second and third irrigation were carried out with the same volume of cyanobacterial suspension four and six weeks after planting. The experiment was completed after 60 days. Vegetative growth parameters including leaf number, fresh and dry weight of leaf, stem length, fresh and dry weight of the plant, root length, fresh and dry weight of root were measured and calculated.

Biochemical analysis

The quality of hormones affecting vegetative growth parameters was determined, with the emphasis on auxin family including indole acetic acid (IAA), indole propionic acid (IPA) and indole butyric acid (IBA) in the extract of studied cyanobacteria. For this purpose, the standard solutions of the studied auxins, namely, IAA, IPA and IBA were prepared by dissolving 1 mg of each standard in 10 ml of water-methanol solution with a ratio of 80:20 (Shariatmadari et al., 2014). Then, the required concentrations were prepared from these stock solutions by successive dilution of 5, 10, 25, 50, 100 and 250 $ng.ml^{-1}$ for calibration curves. The device applied was a high performance liquid chromatography 1100 Model equipped with ultraviolet and fluorescent detectors as well as a HPLC 1200 Model equipped with a photodiode array detector. A wavelength of 225 nm was used in the analysis by using chromatography with a PDA detector. In addition, an excitation wavelength of 280 nm and an emission wavelength of 360 nm were applied with a fluorescence detector. The length of the chromatography column used was 25 cm with an internal diameter of 4.6 mm. Mobile phase was methanol-water and solid phase was Euros Pere 100-5 c18 from KNAUER company.

In order to carry out this part of the study, 21-days cyanobacterial solid culture, *Hapalosiphon welwitschii* and *Anabaena vaginicola* were collected. Then, the specimens were dried completely by a freeze dryer device. In the next step, 10 mg of each cyanobacterium were poured in 1 ml of water-methanol solution with a ratio of 80:20. Then, they were exposed to ultrasonic waves by ultrasonic

bath apparatus at different intervals. These time intervals were 15, 30, 45 and 60 minutes. In the next step, the suspensions were centrifuged and again, 1ml of fresh solvent (water-methanol) was poured in to cyanobacteria. Then the energy was received as the same way at 15-minute intervals, and the extraction operation was repeated. In each analysis, 20µl of the extract was injected into the HPLC apparatus. Then the results of the various chromatograms were compared.

Statistical analysis

Data from different experiments were analyzed using Excel 2007. Statistical analysis was done by one-way ANOVA and Tukey's test using SPSS (ver.16).

Results

Effect of Algal Treatment on Plant vegetative Parameters

In this study, two taxa were collected from the studied sites. The identified taxa were: *Anabaena vaginicola* Fritsch & Rich, (Nostocaceae) and *Hapalosiphon welwitschii* West & GS. West (Stigonemataceae).

Studied plants were treated with these cyanobacteria and control was treated with water following peppermint potting. The vegetative parameters of root, stem and leaf were measured after 60 days. The results showed statistically significant difference between treated plants and control in some vegetative parameters (Table 1). The leaf number as well as fresh and dry weight of leaves in the plants treated with *Anabaena vaginicola* showed significant differences with compared control plants. *Hapalosiphon welwitschii* treatments also showed a significant difference with controls, especially in stem length and fresh weight of root (Figure 1).

Qualitative and quantitative evaluation of metabolites affecting growth with emphasis on auxin family

Using high performance liquid chromatography (HPLC) technique for qualitative identification of the auxin family metabo-

Table 1. Effect of Cyanobacterial suspension on vegetative parameters of peppermint (*Mentha piperita* L.).

	Leaf		Stem			Root		
	Number	F.W	D.W	Length	F.W	D.W	Length	F.W	D.W
Control	545.33±57.83	17.98±1.44	3.427±.33	34.7±1.5	32.03±22.23	5.83±.53	22.5±1.58	20.44±2.94	0.168±0.052
Hapalosiphon	639.33±68.39	22±1.63	4.029±.26	41.47±1.04*	39.23±3.09	7.02±.5	25.34±1.58	34.4±2.94*	0.13±.028
Anabaena	854.33±57.3*	43.63±4.03*	4.96±.54*	36.83±.88	43.63±4.03	8.72±.92	26.47±1.72	26.88±1.72	0.16±.018

DW: Dry Weight, FW: Fresh Weight

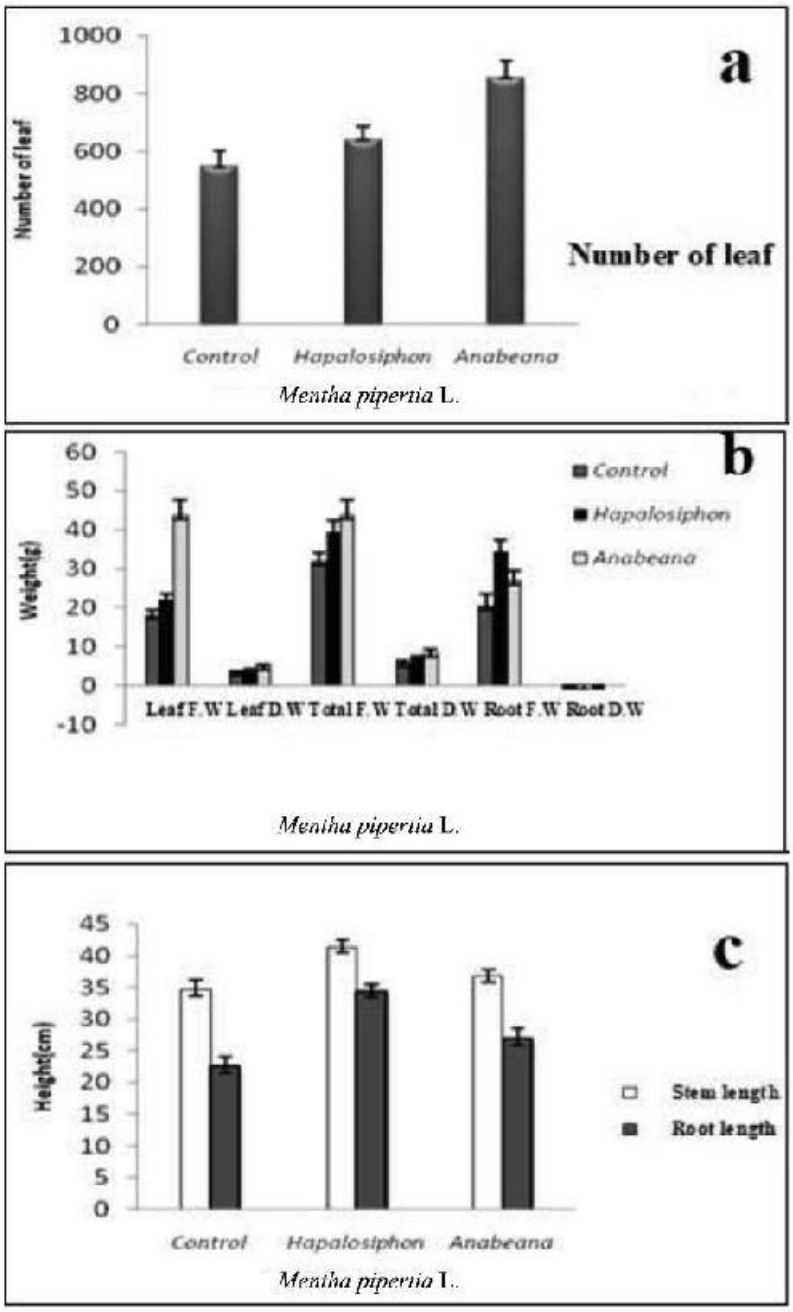

Fig. 1. a) The effect of cyanobacterial suspension on the number of peppermint leaves; b) The effect of cyanobacterial suspension on leaf fresh weight, leaf dry weight, total fresh weight, total dry weight, root fresh weight, and root dry weight; c) The effect of cyanobacterial suspension on the stem length and root length of peppermint.

lites in cyanobacterial biomass of *Hapalosiphon welwitschii* and *Anabaena vaginicola* was performed. The comparison of the obtained chromatograms have been shown that IBA was the most observed hormones in 30 min. (Figs 2 and 3). Therefore, the optimum duration for extraction is 30 minutes. In addition to the IBA, Indole Acetic Acid (IAA)

was also found in the extracts.

The comparison between the levels of IBA and IAA in cyanobacterial extracts showed a significant difference between these two hormones in different cyanobacterial extracts. In other words, the quantitative analyses performed on these two cyanobacterial extracts showed higher levels of IBA, as a

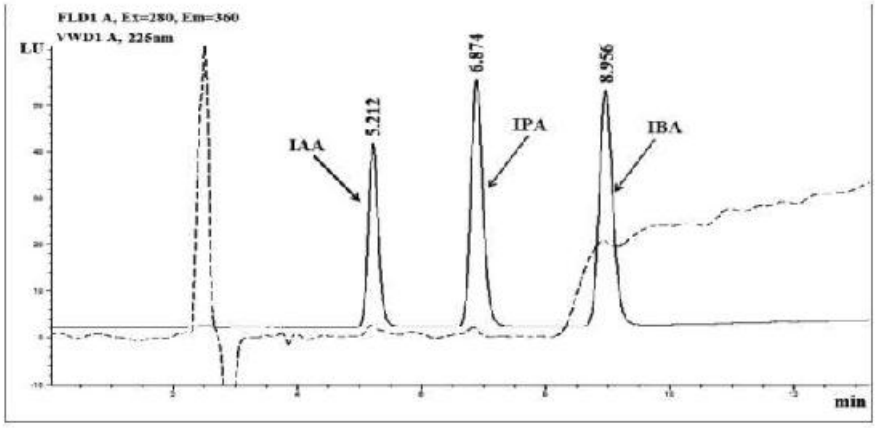

Fig. 2. The chromatogram of isolating four standard compounds of auxin and detection by fluorescence detector.

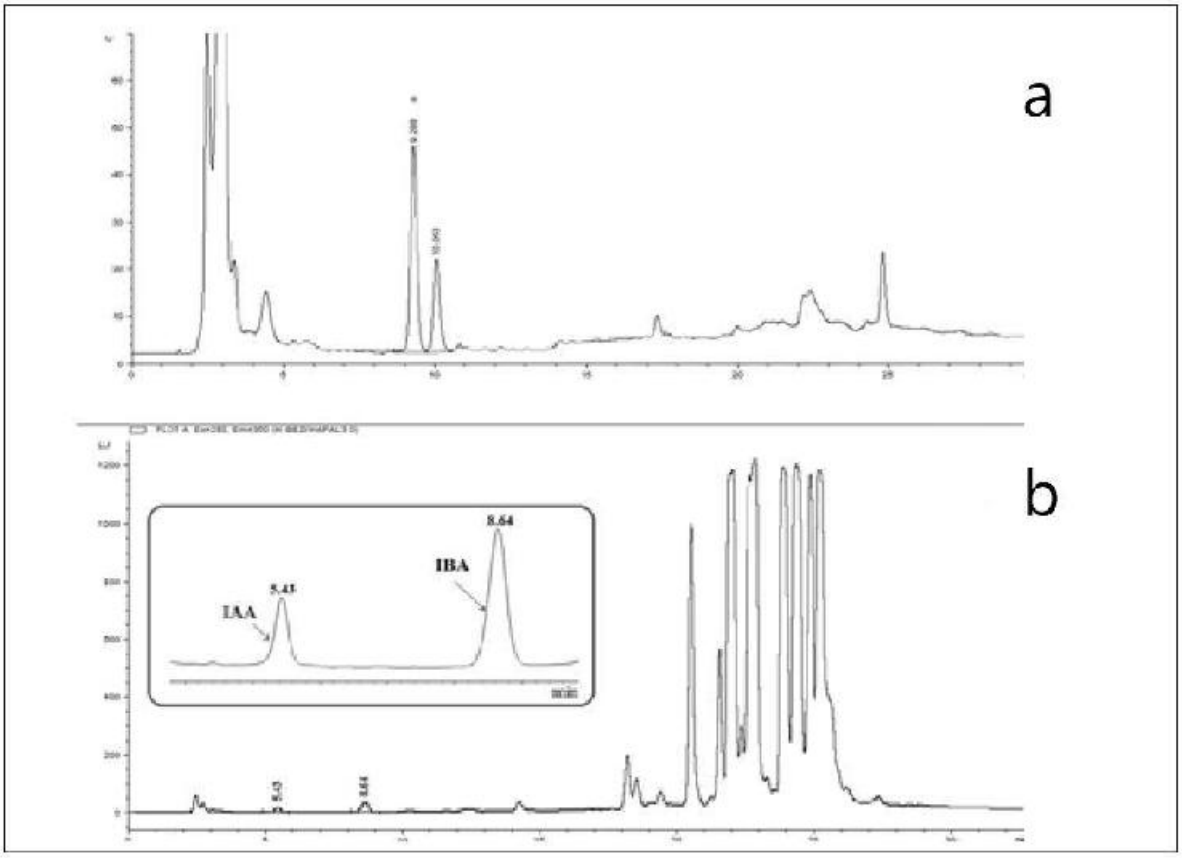

Fig. 3. a) The chromatogram of the ultrasound *Anabaena vaginicola* specimen in methanol-water (20:80) for 30 minutes and detection by the fluorescence detector; b) HPLC chromatogram of *Hapalosiphon welwitschii* specimen extracted by ultrasonic waves for 30 minutes (detected by fluorescence detector).

Table 2. Estimated concentrations of three auxin compounds in the studied Cyanobacteria.

	Estimated Concentration (μg/g) in DW (FW)		
	IAA	**IPA**	**IBA**
Hapalosiphon welwitschii	0.292	-	0.616
Anabaena vaginicola	0.020	-	1.275

growth-promoting hormone, in *Anabaena vaginicola* in comparison with *Hapalosiphon welwitschii* (Table 2).

Discussion

In this study, the effect of cyanobacterial extracts on vegetative growth parameters of medicinal herb, peppermint (*Mentha piperita* L.) was studied. Inoculation of cyanobacterial extract had a positive effect on leaf number, fresh and dry weight of leaf, stem length and fresh weight of root comparing with control. In plants treated by *Anabaena vaginicola*, the maximum growth was observed in leaf characters (leaf number and leaf fresh and dry weight). Plants treated by *Hapalosiphon welwitschii* also showed the maximum growth in parameters such as stem length as well as fresh weight of root.

Over the last few years, researchers observed acceleration of some plants growth by Plant Growth Promoting Rhizobacteria (PGPR) (Asghar et al., 2002; Morissey et al., 2004; Richardson et al., 2009). Soil cyanobacteria have a particular importance in nutrient and organic biogeochemical cycles as well as in maintaining the quality of soil (Kennedy et al., 2004; Khalid et al., 2004). Most studies have been performed on the growth-promoting role of cyanobacteria on *Oryza sativa* L. (Saadatnia and Riahi, 2009). Cyanobacteria are a distinct and primary group of photosynthetic prokaryotes that can supply 86% of nutrient needs of this plant (Venkataraman, 1972). Also, several species of cyanobacteria shown different effects on the vegetative characters of plants such as rice, chick pea, cucumber and squash (Shariatmadari et al., 2011; Prasanna et al., 2008; Yanni and Abd El-Rahman, 1993; Jagannath et al., 2002; Mostafa and Soha, 2009; Tiedemann et al., 1980; Pachpande, 1990; Nanda et al., 1991). Prasanna et al. (2012) reported the performance of metabolites such as IAA derived from cyanobacteria including *Anabaena* sp., *Hapalosiphon* sp., *Nostoc* sp. and *Calothrix* sp. on rice. Mostafa and Soha (2009) used two kinds of fertilizers, *Spirulina platensis* and potassium-humic acid on sesame. They observed that cyanobacterial fertilizer increases plant length, number of branches, number of capsules and weight of the seeds. Some researchers have argued that relationship between cyanobacteria and plants is a mutual relationship for exchanging the nutrients, particularly fixation of carbon or nitrogen by cyanobacteria (Rai and Bergman, 2002; Jaiswal et al., 2008; Karthikeyan et al., 2009). Khanjir (2011) studied the effects of heterocystous cyanobacteria on medicinal herbs, water mint (*Mentha aquatica* L.) and summer savory (*Satureia hortensis* L.). She

justified the incremental effects of cyanobacterial treatments on vegetative parameters and essential oil content with the production of phytohormones by these microorganisms. In addition to studies on growth-promoting effects of cyanobacteria, identification of auxins and their inactive analogues in other algae groups such as Phaeophyceae (*Macrocystis, Laminaria*), Rhodophyta (*Botryocladia*) and Chlorophyceae (*Enteromorpha, Chlorella*) were also performed in 1960s and 1970s (Schiewer, 1967). Similar studies suggest that hormones of the auxin family stimulate formation of rhizoid in green algae *Bryopsis plumose*, and accelerate growth in cyanobacteria and some microalgae (Provasoli and Carlucci, 1974; Arendrachuk, 1974). Auxins are involved in various biological cell cycles, such as cell division, elongation, differentiation and root extension. IAA is known as an important type of auxin which directly affects plant growth (Spaepen et al., 2007). IBA promotes the growth of root cells by stimulating division of the first priming cells. In fact, increasing the concentration of IBA leads to an increase in the number and length of the root. It is due to the effect of this regulator which stimulates adventitious roots and promotes development of latent and pre-formed root primers (Khoshkhooy, 2003).

In this study, the hormones of auxin family (IAA and IBA) which stimulate plant growth, were identified in the extract of *Hapalosiphon welwitschii* and *Anabaena vaginicola*. Due to the higher content of IBA in *Anabaena vaginicola* than *Hapalosiphon welwitshii*, this cyanobacteria were considered as a more appropriate stimulator for treated plants. Therefore, it can be concluded that production of growth stimulating phytohormones can improve growth of treated plants by these cyanobacteria along with the ability to nitrogen fixation. However, it should be considered that other factors such as increasing in organic content of soil, exchanges between cyanobacteria and plants, improving aggregation and soil structure can also be effective.

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