Cyanobacterial Culture as a Liquid Supplement for White Button Mushroom (*Agaricus bisporus*)

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Abstract

Cyanobacteria comprise a large group of structurally complex and ecologically significant gram-negative prokaryotes. There are many reports that these microorganisms produce phytohormones such as cytokinin, auxin and auxin-like substances in soil. Until now most research and applications of cyanobacteria have been conducted with green plants growing. There is no evidence to provide that cyanobacterial culture could stimulate mushroom growth and mushroom yield. In this study, algal culture of heterocystous cyanobacteria *Anabaena vaginicola* Fritsch et ich was sprayed on casing soil at primordia formation stage of *Agaricus bisporus*, before the first, the second and the third flushes. The result showed that inoculation of cyanobacteria into the casing soil as biofertilizer significantly increased mushroom yield and quality. In complementary studies, nitrogenase activity of this alga was determined by acetylene reduction technique and identification of phytohormones was performed with HPLC method. Chemical content of algal suspension was another factor that was analyzed in this experiment.

Keywords: *Anabaena vaginicola,* Casing Soil, Phytohormone, White Button mushroom, HPLC.

Introduction

Agaricus bisporus is one of the most commonly and widely consumed mushrooms in many countries such as Iran. The commercial cultivation of the *Agaricus bisporus* involves two-step processes in accordance with growth stage. The first phase is started by inoculation compost with cereal grains colonized with mushroom mycelium and in the second phase, the development of fruit bodies or sporophore of this mushroom occurred in casing layer of peat and carbonate on top of the colonized compost (Straatsma et al., 1991). It has been reported that the formation of sporophore of *Agaricus bisporus* in the second phase is affected by physic-chemical properties of culture beds and environmental factors such as temperature, CO_2 and relative humidity. Biological characteristics of the casing soil are another group of factors that can affect the production and productivity of *A. bisporus* (Singh et al., 2000). Soil microorganisms, especially bacteria, are the main component of culture beds, some of which has the ability to increase mushroom yield and quality (Ebadi et al., 2012). Not only in *Agaricus bisporus*, but also in the other mushrooms such as *Pleurotus*, addition of bacterial culture to the mushroom growing media

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made mushroom mycelia run faster (Kim et al., 2008). These effective bacteria produce certain metabolites which could initiate sporophore formation. Phytohormones such as Indole 3-acetic acid are the most important factors, which are suggested for growth stimulating effect of these microorganisms. Cyanobacteria or blue-green algae (BGA) are another group of soil microorganisms which can affect mycelial growth of mushrooms**.** Cyanobacteria produce a wide array of secondary metabolites including antibiotics, algicides, toxins, pharmaceuticals and plant growth regulators. Among the growth regulators, gibberellin, auxin, cytokinin, ethylene, abscisic acid and jasmonic acid have been detected in cyanobacteria (Gupta and Agarwal, 1973; Stirk et al., 1996; Ordog and Pulz, 1996; Manickavelu et al., 2006; Seyed Hashtroudi et al., 2012).

There is accumulating evidence that cyanobacteria have been used in agriculture and horticulture as a source of plant growth stimulating agents. Hartung (2010), reported that the extracts of cyanobacteria contain an assembly of beneficial compounds which can be used for special plant treatments, e.g. to decrease plant senescence and transpiration; to increase germination rate of seeds, pod set, leaf chlorophyll content, and seed protein content; and also to enhance the root and shoot development of treated plants. The effects of cyanobacterial inoculation were reported for several groups of higher plants (Venkataraman, 1972; Rodgers et al.,1979; Singh, 1988; Arif et al., 1995; Thajuddin and Subramanian, 2005; Karthikeyan et al., 2007; Maqubela et al., 2008; Saadatnia and Riahi, 2009), but their beneficial influence on other crops such as edible mushrooms is little known, whereas these crops are the most important component of many countries diet. The findings of this research strongly supported the production of promoting substances by cyanobacteria for the greater mushroom growth and yield. Until now there is no evidence to provide that cyanobacterial culture could stimulate mushroom growth and mushroom yield. However, this is the initial attempt to irrigate mushroom casing soil with the algal culture and still needs further investigation.

Materials and methods

Blue-Green Algae (Cyanobacteria) Heterocystous

cyanobacteria, *Anabaena vaginicola* Fritsch et Rich (ISC90), was obtained from Botany Department, Faculty of Biosciences, Shahid Behashti University. An axenic culture of cyanobacteria was prepared by inoculating these algae on nitrate free BG-11 medium in a five liter container at 25°C and a 12/12 h light-dark cycle at artificial illumination (4,000 to 4,500µmol photons $m^{-2}s^{-1}$) with constant stirring and aeration. After four weeks, the culture was harvested (Optical density: 0.3) and used as an inoculum. Algal culture was sprayed on the casing soil at primordia formation stage, before the first, the second and the third flushes.

Mushroom Production

The compost formulation consisted of wheat straw, chicken manure and gypsum prepared in conventional yard and pasteurization tunnels. Mushrooms were grown in controlled and standard growing room. After colonization of compost with *A. bisporus* mycelium (commercial strain Sylvan A15), the substrate was covered with 4-5cm of casing soil. Mushroom yield was

determined over a 3-weeks production period. Mushrooms were weighted after removing the stipes.

Identification and Quantification of Phytohormones: Preparation of auxin standards and extraction procedure

Identification and quantification of the endogenous auxins was performed according to Seyed Hashtroudi et al. (2012). The stock solutions were prepared by dissolving 1mg of each auxin (IAA, IBA and IPA) in 10ml of methanol separately and also as a mixture and subsequent dilutions were made with methanol-water 80:20 to prepare the desired concentrations. Algal biomass was dried, using an Operon bench top freeze dryer (FDB-5503) and extraction was performed under the commonly used sonication conditions of 20°C for the optimized time of 30min. The extraction solvents were methanol-water in a ratio of 80:20. The extracts were centrifuged in 7500 rpm for 10min; the supernatant was filtered through 0.45µm syringe filter and concentrated to 500-1000µl using an Organomation N-EVAP.

HPLC performance

Chromatographic separation was performed on an Agilent 1200 series HPLC system including a quaternary pump and a degasser equipped with a G1315D Diode Array Detector and a G1321A Fluorescence Detector. The accompanying Agilent LC Chemstation was employed for instrument control, data acquisition and processing. A Eurosphere RP-column (100-5 C18 column, 250×4.6mm Knauer, Germany) was used for separation of analyses. The column was eluted with a linear gradient (0–5 min, 60% A, 5-20min, 100%A) at a flow-rate of 1mlmin^{-1} of methanol (A) and 0.3% AcOH and the column temperature was maintained at 25°C. Considering the UV maxima of three auxins, UV detection was performed at 225nm and excitation and emission wavelengths in the fluorescence detector were 280 and 360nm, respectively.

Both the extracts and standards were injected (injection volume: 20µl) into the reverse phase column and identifications were carried out using comparison of retention times and UV spectrums of the extracts with standard mixture. Each experiment was repeated at least three times and run in triplicate. Recoveries were calculated by adding a known amount of standards to the microalgae and extracting the auxins with the same method as described above.

Nitrogenase activity measurements

Nitrogenase activity was determined by acetylene reduction technique according to Asadi et al. (2011). Prior to incubation, 10% of the air inside the vial was replaced with the same volume of acetylene. Cells were incubated for 1 hour under the same conditions as they were cultured. After incubation, 0.5mL of gas samples was taken and ethylene concentration was determined. Acetylene reducing activity was determined on a Shimadzu GC-15A gas chromatograph as nmol ethylene mg dry weight^{-1} hour^{-1}.

Evaluation of chemical contents of algal extract

Chemical content of the algal extract, such as total nitrogen and inorganic nitrogen (NO_2^-, NO_3^- and NH_4^+), phosphate, sulfate, carbonate and cations (Na^+, K^+, Mg^{2+}, Ca^{2+}) were determined by Arian Fan Azma Institute, Tehran, Iran. Laboratory methods of measurements are

summarized in Table 3.

Statistical Analysis

An analysis was performed with independent samples t-test and significant differences were surveyed at 0.05 level. A completely randomized design was used with four replications for each treatment.

Results

In order to explore the cyanobacteria effect on edible mushrooms, one heterocystous cyanobacteria, *Anabaena vaginicola* ISC90, was used as a biofertilizer for *Agaricus bisporus*. The casing soil of treated mushrooms was irrigated with algal culture at the pining or primordia formation stage of the first, the second and the third flushes. There was a significant difference in mushroom yield in three picking treated with cyanobacterial culture as compared to the control (Fig. 2). The mushroom yield increased 0.661 kg/m^2 in the first flush, 2.2 kg/m^2 in the second flush and 0.053 kg/m^2 in the third flush as compared to the control. The result also showed that inoculation of casing soil with cyanobacteria had a positive effect on the quality of mushroom. Dry matter content of mushrooms increased in the first flush. Dry matter was measured at 9.75% in treated and 8.35% in untreated mushrooms. However, the amount of dry matter and protein declined in the second and the third flushes as compared to the first flush. There was a slight difference in dry matter in treated and untreated mushrooms in the second and third flushes. Addition of algal culture also had a positive effect on protein content of treated mushrooms. The protein content increased significantly in the first flush, but there was a slight difference between treated and controls in the second flush (Table 1)

Many reports have been published on the stimulating effects of macroalgal extracts on the growth and development of higher plants. Indole 3-acetic acid (IAA), one of the main growth regulators of higher plants, belongs to the substances detected from these algal extracts (Mazur et al., 2001). Not only macroalgae but some of microalgae such as cyanobacteria are used as a source of plant growth-promoting substances. Due to the critical role of auxins in the mushrooms growth, synthesis of three major natural auxins, namely indole 3-acetic acid (IAA), indole 3-propionic acid (IPA) and indole 3-butyric acid by our native cyanobacteria strain (*Anabaena vaginicola* ISC90) was investigated. The results of using high performance liquid chromatography equipped with fluorescence detector showed the presence of these compounds, especially Indole 3-butyric acid (IBA) and Indole 3-acetic acid (IAA), in the cyanobactrial biomass (Table 2).

The HPLC chromatograms of the *Anabaena vaginicola* ISC90 sample and standard mixture of three auxins under the optimized HPLC conditions are shown in Figure 1. The peaks were identified by comparing the retention times of authentic standards and also spiking the individual standards to the microalgal extract. Measurement of nitrogenase activity of this alga also showed that this alga has the ability to fix nitrogen in natural condition (Table 2). Chemical content of algal extract as well as production of stimulating factors such as phytohormones were the most important factors that may affect the growth of treated mushrooms in this experiment (Tables 3).

Discussion

Agaricus bisporus or white button mushroom is an edible fleshy fungus which has approximately 1.5 million tons annual production in Iran. Mushroom production depends on some physico-chemical and biological properties of culture beds. The casing layer is a major source of variation in yield, quality and uniformity of commercial cropping (Noble and Gaze, 1995). The results of this study showed that irrigation of casing soil with cyanobacterial culture increased yield, dry matter and protein content of *Agaricus bisporus*. Before this study, the positive effect of plant growth promoting bacteria on growth parameters of *Agaricus bisporus* and the amounts of mushrooms nutrients was reported by Ebadi et al. (2012).

They showed that *Agaricus bisporus* yield increases due to inoculation mushroom culture bed with *Rhizobium* and *Psudomonas* strains.

Table 1. Effect of algal culture on yield, dry matter and protein content of mushroom

Sample	First Flash			Second Flash			Third Flash		
	Yield Kg/m^2	D.M %	Protein %	Yield Kg/m^2	D.M %	Protein %	Yield Kg/m^2	D.M %	Protein %
Control	12.589	8.35	48.1	7.582	8.3	45.1	2.747	7.40	41.1
Test	13.250*	9.75**	51.9**	7.380**	8.6*	45.5ns	2.800ns	7.50ns	39.1ns
Diff.	0.661	1.4	3.8	0.202	0.3	0.4	0.053	0.10	-2.0

Table 2. Nitrogenase activity and estimated concentrations of three auxins in the microalgal samples

Microalgae	Nitrogenase activity (nmol/mg h)	Estimated Concentration (µg/g) in DW		
		IAA	IPA	IBA
Anabaena vaginicola Fritsch et Rich	896.0	0.020	nd	1.275

Table 3. Chemical contents of *Anabaena vaginicola* ISC90 extract (1% water extract).

	Analytical Method	*Anabaena* extract
pH	Electrometric	6.62
EC (µS/cm)	Platinum Electrode	63.00
Total Nitrogen (mgL^{-1})	Macro kjeldahl	162.00
NO_2^- (mgL^{-1})	Colorimetric	0.02
NO_3^- (mgL^{-1})	Ultraviolet Spectrophotometric	<1.00
NH_4^+ (mgL^{-1})	Nesslerization	80.20
Phosphate (mgL^{-1})	Vanadomolybdophosphoric acid colorimetric	10.40
SO_4^{2-}(mgL^{-1})	Gravimetric	50.00
CO_3^{2-}(mgL^{-1})	Titrimetric	0.00
HCO_3^- (mgL^{-1})	Titrimetric	250.00
Ca^{2+} (mgL^{-1})	EDTA Titrimetric	280.00
Mg^{2+} (mgL^{-1})	EDTA Titrimetric	0.00
Na^+ (mgL^{-1})	Flame Emission Photometric	12.00
K^+ (mgL^{-1})	Flame Emission Photometric	6.10

They also reported that some of these strains had the ability to produce IAA. The positive effect of inoculation of the edible and medicinal mushrooms with growth regulators such as auxins has been reported both in vitro and in vivo (Shukla, 1995; Kaur and Lakhanpal, 1995; Guo et al., 2009; Theradimani et al., 2001). Increase in biomass production as well as protein content of the mycelium in the edible mushroom, *Pleurotussajor-caju* by phytohormones was also reported by Mukhopadhyay et al. (2005). They showed that maximum enhancement of mushroom growth and protein content in this

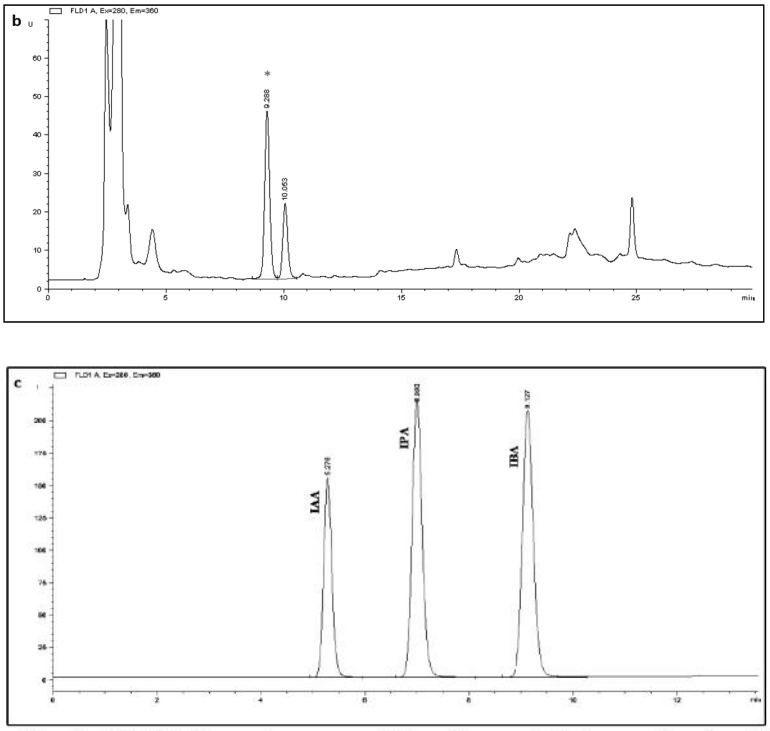

Fig. 1. HPLC chromatograms of the ultrasonicated samples for 30 min. (b) *Anabaena vaginicola,* (c) Standards

Fig. 2. Effect of *Anabaena vaginicola* inoculum on the *Agaricus bisporus* yield as compared to the control

edible mushroom was observed with indole 3-acetic acid.

The results of using high performance liquid chromatography equipped with fluorescence detector in the present study showed the presence of these compounds, especially Indole 3-acetic acid (IAA) and Indole 3-butyric acid (IBA), in *Anabaena vaginicola* ISC90 biomass (Fig. 1). The dominant auxin observed in this isolate was IBA and only trace amount of IAA was detected. The results of this study emphasized the importance of auxins in the increase of *Agaricus bisporus* yield and quality. In other words, the results revealed that these phytohormones not only increase plant growth and plant biomass, but they could positively affect the mushroom yield and quality. The results also showed that cyanobacterium *Anabaena vaginicola* ISC90 is a producer of phytohormones which can stimulate mycelial growth and quality in commercial production of *Agaricus bisporus*. Before this study, Shariatmadari et al. (2012) reported heterocystous cyanobacteria, *Anabaena vaginicola* ISC90 and *Nostoc calcicola*, have the ability to promote vegetable growth and they are appropriate candidates for the formulation of a biofertilizer for higher plants. They suggested that increasing the essential micro-elements in soil, which are necessary for plant growth and plant ion uptake, and nitrogen content of the surface soil as well as production of plant growth promoting substances, by these microorganisms, are the most important factors that can stimulate plant growth (Shariatmadari et al., 2012). A positive effect of cyanobacterial culture on mushroom yield and quality was not previously reported and this is the initial attempt to irrigate mushroom casing soil with algal culture. In the present study, chemical content of algae extracts and production of plant growth-stimulating substances such as phytohormonesareare proposed as factors affecting mushroom yield and quality but unfortunately not sufficient information is available around the requirement of micro-elements for *A*. *bisporus* (Fermor et al., 2000).

In this study, we reported the positive effect of cyanobacteria inoculation on yield, dry matter and protein content of *Agaricus bisporus*. Production of promoting substances such as phytohormones is one of the main factors suggested for this issue. Study of HPLC application strongly supported that the production of two major natural auxins, namely indole 3-acetic acid (IAA) and indole 3-butyric acid (IBA) by cyanobacteria that not only increases plant growth and enhances plant biomass but it could positively affect the mushroom yield and quality. However, this is the initial attempt to irrigate mushroom casing soil with algal culture and still needs further investigation.

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