Two Main Tropane Alkaloids Variations of Black Henbane (*Hyoscyamus niger*) Under PGPRs Inoculation and Water Deficit Stress Induction at Flowering Stage

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Abstract

Background: Plants infection with microorganisms as well as physical factors such as osmotic stresses especially drought stress induced particular secondary metabolite production.

Objective: Plant root and shoot hyoscyamine (HYO) and scopolamine (SCO) variations were investigated under three water deficit stress (WDS) levels as 30 (W1), 60 (W2) and 90 % (W3) water depletion of field capacity and *Pseudomonas putida* (PP) and *P. fluoresence* (PF) strains inoculation at flowering stage.

Methods: Seedling radicles and culture media were inoculated with PP and PF (10^9 CFU/ml) strains. Monitoring of soil moisture levels was conducted using gravimetric method. Alkaloids extracted were identified by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis.

Results: Results revealed that HYO was the prevalent alkaloid in root and shoot organs. The highest ratio of SCO to HYO (0.53) and the highest alkaloids both HYO and SCO content in root and shoot was observed in W3PF treatment. The largest total alkaloids (HYO+SCO) yield (25.7 mg.plant^{-1}) was showed in PP treated plants under W1 conditions.

Conclusion: Integrative use of effective *Pseudomonades* strains and WDS sounds to be an encouraging and eco-friendly strategy for increasing tropane alkaloids yield and content in *Hyoscyamus niger* root and shoot parts.

Keywords: *Hyoscyamus niger*, PGPR (Plant Growth Promoting Rhizobacteria), Tropane alkaloids, Water deficit stress

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Introduction

Black henbane (*Hyoscyamus niger*) is a herbaceous medicinal plant and one of the most important commercial source of pharmaceutical tropane alkaloids in the family of Solanaceae. This plant is of interest due to its production of bioactive tropane alkaloids, including scopolamine (SCO) and hyoscyamine (HYO), which are generally used as anticholinergic, antispasmodic, mildly pain-relieving, hypnotic, hallucinogenic, pupil-dilating and sedative [1]. Because of the complex chemical structures of these alkaloids, industrial synthesis has been found to be prohibitively expensive and therefore they are mainly obtained from plant resources of Solanaceae family [2, 3]. Also, these metabolites have been reported in other plant families, e.g. Orchidaceae, Euphorbiaceae, Cruciferae, Convolvulaceae [4]. Plant fine roots without secondary growth have been found to be the principal site of tropane alkaloids production, where the main enzymes of their biosynthetic pathway are localized [5]. Plants infection with microorganisms as well as physical factors such as osmotic stresses induced particular secondary metabolite pathways [6]. Among the numerous microorganisms in rhizosphere, some have positive effects on plant growth promotion comprising plant growth promoting rhizobacteria (PGPR) such as *Pseudomonads* strains, which colonize the rhizosphere and roots of many plant species and confer beneficial effects to plants. Although, drought stress negatively affect the growth and development of field crops, the contents of secondary metabolites are mostly increased through the positive effects on the metabolic pathways of active compounds synthesis in medicinal plants [7]. Inoculation of plants with native suitable microorganisms may decrease deleterious effects of environmental stresses and increase stress tolerance of plants by a variety of mechanisms including synthesis of phytohormones such as auxins [8], solubilization of minerals like phosphorus, production of siderophores [9], increases in nutrient uptake, leaf area, chlorophyll and soluble leaf protein content [10]. In recent years, many experiments have been conducted on the role of microbial association like *Arbuscular mycorrhizal* symbiosis and PGPRs in drought stressed crop plants [11].

However, there have been no comparative studies about influence of *Pseudomonades* strains such as *Pseudomonas putida* (PP) and *P. fluorescence* (PF) under water deficit stress (WDS) on tropane alkaloids in such a commercially important medicinal plant. Also, no investigations have been performed regarding alkaloids yield and entire content of these metabolites on a whole plant basis under respective treatments. The objectives of this study were: [1] to study the effects of twenty *P. putida* and *P. fluorescence* strains on early growth promoting of *Hyoscyamus niger* in terms of seedling vigor index, and [2] to investigate the effects of two most efficient strains on *H. niger* root and shoot tropane alkaloids (HYO, SCO) variations under water deficit stress conditions.

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Materials and methods

Seed preparation

Seeds of *Hyoscyamus niger* were provided by Agricultural and Natural Resources Center, Esfahan, Iran. Black Henbane seeds generally have low germination rate under normal laboratory conditions. Therefore, seeds were treated with 250 mg.l gibberellic acid (GA_3) for 48 h at room temperature (25 ± 0.5˚C) for breaking dormancy and accelerating germination. After that seeds were surface-sterilized in 70 % ethanol for 2 min and then in 25 % commercial bleach (containing 6 % sodium hypochlorite) for 10 min and finally rinsed with sterile distilled water. Subsequently, seeds were placed in petri dishes on two layers of filter paper (Whatman No. 1) moistened with 4 ml distilled water. After 3 days, 90% of seeds germinated steadily (with 1 - 2 mm radicle length).

Bacterial strains and inoculation

PGPR strains (Kindly provided from soil and water research institute, Karaj, Iran) with different abilities in auxin production were used for laboratory experiment (Table 1). To prepare inoculums, a single colony of each PGPR strain was transferred to 100 ml flasks containing 25 ml of TSB (tryptone soybean broth) and grown aerobically in flasks on a rotating shaker (120 rpm) for 72 h at 28°C. The bacterial suspension was then diluted in sterile distilled water to achieve a final concentration of 10^9 CFU/ml. The prepared suspensions were used to inoculate henbane radicles and culture media under aseptic conditions.

Table 1- Mean auxin (IAA) production (mg.L^{-1} ± 0.01) capability of the *Pseudomonas putida* and *P. fluorescens* strains in the absence of L-TRP (tryptophan)

***Pseudomonas fluorescens* strains**	**Mean IAA production (mg/l)**	***Pseudomonas Putida* strains**	**Mean IAA production (mg/l)**
4 Akhgar	2.380	4	8.499
12Akhgar	1.200	11	6.752
73	2.960	41	47.694
174	7.350	53	30.126
99	13.388	108	9.774
169	5.775	112	31.222
173	12.470	143	32.941
187	5.152	147	29.030
196	6.130	159	76.697
CHAO	*	168	17.921

*Variable in different culture media

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Selection of two most effective strains under plate and tube assay

Plate assay *(in vitro*)

For plate experiments seedlings radicles were inoculated with the corresponding bacterial strains. For this purpose, ten pre-germinated seeds after treatment were planted in the petri plates containing water agar (1.5%) and then placed on incubator (23 ± 1°C). After 15 days, the length and weight values were measured for root and shoot of individual seedlings to determine the vigor index (1 and 2) using the method described by Abdul Baki and Anderson [12]. *VI (1) = (RL+SL) × GP (%)* and VI *(2) = (RDW+SDW) ×GP (%)*. Where VI is vigor index, RL is root length (cm), SL is shoot length (cm), RDW is root dry weight (g), SDW is shoot dry weight (g) and GP is germination percentage which is 100% for all treatments because uniformly germinated seeds were used in this study. Untreated radicle and culture media served as control (C). Sterilized broth (free of bacterial population) was applied in case of control plus TSB medium (C+M).

Tube assay (growth room)

After inoculation, two seedlings with 1 – 2 mm long radicle were transferred to the holes made at the top of sterilized plastic tubes (3 cm in diameter and 12 cm height). Tubes were filled with 120 g acid washed sand and 0.5 ml of rhizobacterial strain was added into two holes before planting (both radicle and sand inoculation). The tubes were then incubated in a vertical position in wooden boxes in growth room at 24 ± 2°C with 14 - 10 h illumination and dark, respectively. Sterile water was supplied daily to maintain constant water content. After 30 days, different plant parameters were measured. The total root length was determined using the gridline intersect method [13]. The experiment was arranged in a randomized complete block design with three replicates for twelve treatments in each genus of *Pseudomonades* strain, as follow:

PF strains: 4 Akhgar, 12 Akhgar, 73, 174, 99, 169, 173, 187, 196, CHAO, C and C+M.

PP strains: 4, 11, 41, 53, 108, 112, 143, 147, 159, 168, C and C+M.

Plant growth conditions and water deficit stress induction

Based upon the efficiency of rhizobacteria in enhancing the seedling vigor index (from results of *in vitro* and tube assays), two of the most effective PGPR strains with different multiple plant growth promoting activities namely PP-168 and PF-187 were selected and used in greenhouse study (Table 2). The inoculums suspensions of these two PGPR were applied on both seedlings radicles (1 - 2 mm length) and evenly within the two holes (1 cm depth) on the pot soil surface at a rate of 0.5 ml per seedling using syringe. Thereafter, two inoculated seedlings were immediately transplanted to the each plastic pot (25 cm diameter and 30 cm deep) containing 8 kg soil, which sterilized by autoclaving at 105◦C for 60 min over three consecutive days. Untreated radicles and culture media served as control (C). Sterilized Broth (free of bacterial population) was applied in the case of control

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Table 2- Multiple plant growth promoting activities of *Pseudomonas putida*-168 (PP) and *Pseudomonas fluorescens - 187* (PF) strains

Pseudomonas strains	PGPR activities				Ecological site of strains isolation (rhizosphere type)
	P solubility ($\mu g.ml^{-1}$)	Siderophore production	IAA production* ($mg.l^{-1}$)	HCN Production score	
PP- 168	-	2.25	17.921	5 (high)	Wheat (cv. Gaskozhen**)
PF-187	438.38	1.25	5.152	0	Wheat (cv. Azar***)

PGPR: Plant Growth Promoting Rhizobacteria, * IAA: Indole-3- acetic acid (without presence of tryptophan), P (phosphorus), **Irrigated variety (Hydrophyl), ***Dry land variety (Xerophyl), HCN: Hydrogen Cyanide

plants. The experiment was conducted in a greenhouse located in faulty of agricultural science, Tehran University (Karaj, Iran). During the experiment, the temperature ranged from 26◦C to 30◦C, and the relative humidity was between 65% and 75%. Plants were subjected to WDS treatments from 45 days after planting for 60 days. The field capacity (FC) of soil was determined 12.5% using the pressure plate apparatus. The pot surface was covered by sterile plastic beads to minimize evaporation and avoid contamination. Estimation of water depletion levels was done through weighing pots daily (morning hours). Monitoring of soil moisture levels was conducted using gravimetric method [14]. The mechanical and chemical composition of the soil was as follows: 58.4% sand, 18.8% silt, 22.8 % clay; total available N; 0.12%, P; 8.14 ppm, K; 175 ppm. The study was set up as randomized complete block design (RCBD) with factorial treatments of three watering frequencies (30, 60, and 90% depletion of FC) and two PGPR strains along with control (without PGPR inoculation) in three replications. All the pots were kept to the field capacity up to 45 days after planting. After 60 days of WDS all pots were harvested at the full flowering stage. Plants were uprooted, washed carefully, and then shade dried, finely powdered in an electronic blender and kept in separate containers for tropane alkaloid extraction.

Alkaloid extraction

Plant samples (root and shoot) were sieved with laboratory mesh (size 30, mesh opening 545 μm). A subsample of 2 grams from each samples was added to appropriate volume of $CHCl_3$: MeOH: NH_4OH 25%, (15:5: 1), sonicated for 20 min and then kept at water bath (40◦C) for one hour. Subsequent sample preparation and alkaloids extraction were based essentially on the method described by Kamada et al. [15].

Alkaloid analysis and quantification

Alkaloids extracted were identified by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis. Gas

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chromatography analysis was performed using a GC system equipped with a flame ionization detector (FID) and HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm). Injector and detector temperatures were set at 220 and 290 ◦C, respectively. The column temperature was initially kept at 50 ◦C for 5 min, then gradually increased to 300 ◦C at a rate of 3ºC/min and maintained for 3 min. The flow rate of gas helium was 0.8 mL/min. Then 1 µL of extract was directly injected into the gas chromatograph. Each extraction was replicated three times and the compound percentages are the means of the three replicates. GC-MS analysis was carried out on an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, USA) fitted with a fused silica HP-5MS capillary column (30 m×0.25 mm×0.25 µm). Oven temperature was programmed from 50ºC to 285ºC at 3ºC/min, and helium was used as carrier gas (0.8 mL/min), Mass spectra were obtained in an Agilent 5973 system operating in electron impact mode (EIMS) at 70 eV, coupled to an GC system. The identification of alkaloids was based on the comparison of their GC retention time and mass spectra (MS) data with their standards substances (HYO. HCl and SCO. HBr, Merck). The total tropane alkaloids (HYO + SCO) yield was quantified by both alkaloid content and biomass production; Total alkaloid yield (mg.plant^{-1}) = Alkaloid content (% d.w) × Plant dry weight (mg.plant^{-1}).

Statistical analysis

All analyses were performed based on a randomized complete block design (RCBD) with factorial treatments. The data were subjected to ANOVA and were analyzed by using SAS program, and probabilities of significance were used to test for significance among treatments and interactions, and lowest standard deviations (LSD) test were used to compare means ($p<0.05$). Values obtained were expressed as mean ± SD (standard deviation) from three replications (n=3) of each treatment.

Results

Selection of two most effective strains

Analysis of variance from laboratory experiments indicated that different strains of rhizobacteria varied significantly in their efficiency for enhancing the weight and length of root, shoot and subsequently seedling VI. The same trend was observed for both VI 1 and VI 2 with various PP and PF strains under plate and tube assays, hence only the results of VI 1 is presented (Figure 1). Results showed that different strains of rhizobacteria had variable effects (both negative and positive) on VI in two various tested assays. Among 10 PF strains, which were used in plate assay, strains 196 and then187 were the best ones on enhancement of VI (Figure 1A). All these strains were further tested in tube experiment, which showed that all strains except CHAO increased VI. For example, strain 187 increased the VI up to 72% compared with uninoculated control (C) (Figure 1B). PF-187 strain increased root and shoot elongation by 73% and 51%, respectively compared with uninoculated control (data not shown). In plate

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assay, PP-159 and PP-168 strains had the highest values of VI (Figure 1C) but in the tube experiment only PP-168 strain was the superior one, which enhanced VI up to 62% over untreated control (Figure 1D). The maximum increase in root and shoot length (63% and 46%) was recorded for PP-168 strain. Two strains of PP (4 and 11) had negative effects on VI.

Tropane alkaloids variations

Tropane alkaloid biosynthesis in solanaceous plants alters dynamically with plant developmental stages and growth conditions. Our results showed significant differences between content and yield of two mains tropane alkaloids including HYO and

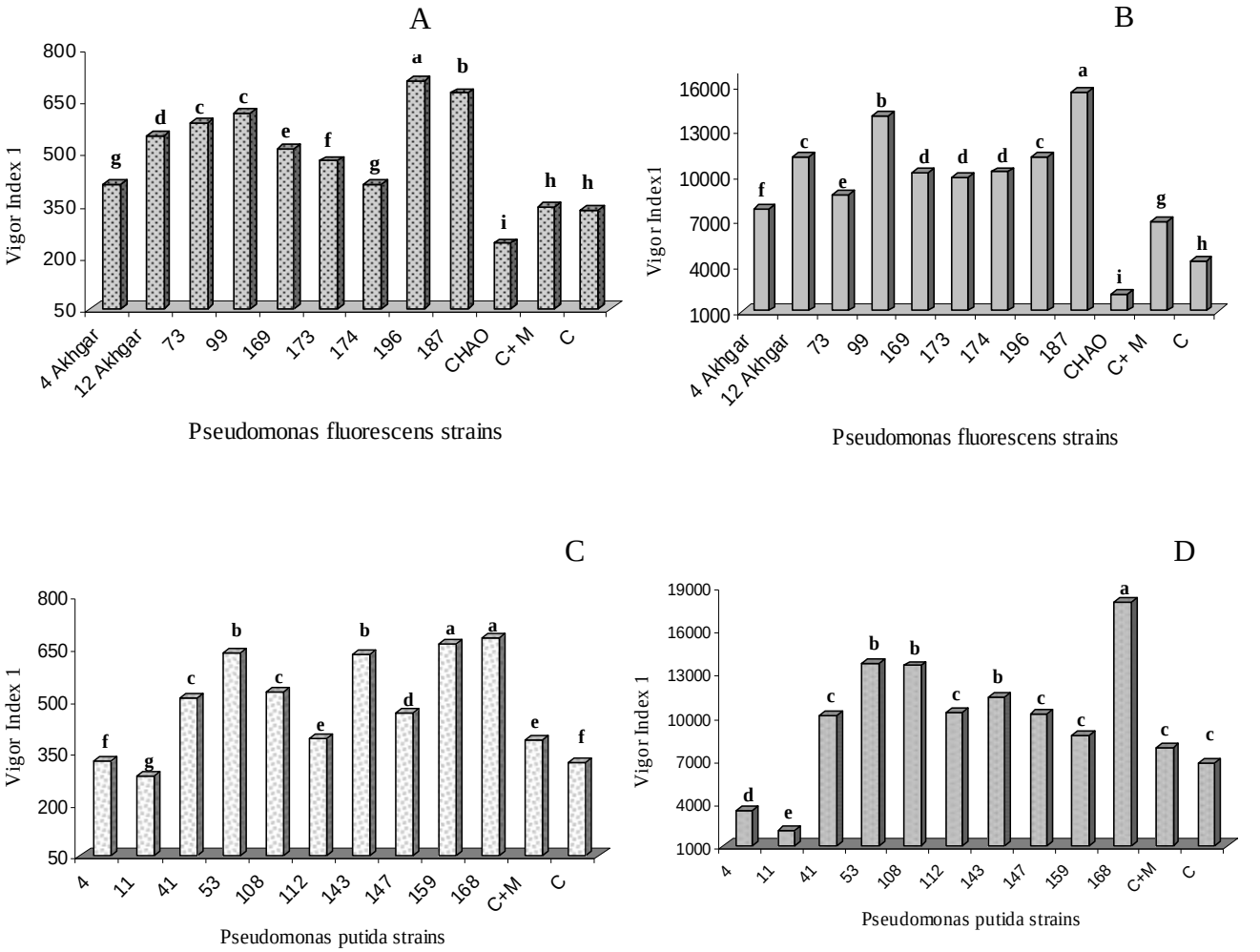

Figure 1- Effect of *pseudomonas fluorescens* and *Putida* strains inoculation on *hyoscyamus niger* seedling vigor index 1 in plate (A and C) and tube (B and D) assays. C and C+M in horizontal axis refers to control and control plus bacterial medium (TSB) treatment, respectively. Values marked with the same letter are not statistically different

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SCO at vegetative growth stage [16] and full flowering time (this study) under PGPRs inoculation and water stress conditions. In PGPRs treated and un-inoculated control plants, SCO content of roots were increased significantly with increasing WDS up to W2 treatment, and later it started to decline, except for PF treated plants, which kept continuously upward trend (Figure 2). The largest root SCO content (0.133% DW) was observed in the PF treated plants under W3 conditions. There was no significant interaction among all treatments on root HYO content, but influenced by PGPRs (Figure 3a) and WDS (Figure 3b) separately. The maximum root HYO content showed in W3 treatment, while both PP and PF strains effects were identical. In shoots, however, HYO content significantly increased with increasing WDS in all employed treatments (Figure 4). Results also showed that HYO content of shoots under PF and PP inoculation plants were almost 14% and 10% higher than that of control plants, respectively. SCO content of shoots in all employed treatments had same changes as root, and was mildly increased with increasing WDS only under PF treated plants (Figure 4). It seems that inoculation of *H. niger* plants with PF strain promoted HYO and SCO accumulation in both root and shoot organs. Almost the same trend was observed for both root and shoot alkaloids (HYO and SCO) yield variations under all employed treatments, hence only the results of shoot tissue were presented (Figure 5). Shoot HYO yield was decreased with increasing WDS in both PGPRs treated and uninoculated control plants. However, the reduction percentage in PP and PF treated plants was lower than un-inoculated control. Shoot SCO yield also decreased with increasing WDS in PP treated and control plants, but in PF treated plants showed unchanged (Figure 5). The largest total alkaloids (HYO+SCO) yield (25.7 mg.plant^{-1}) showed in PP treated plants under W1 conditions (Figure 6).

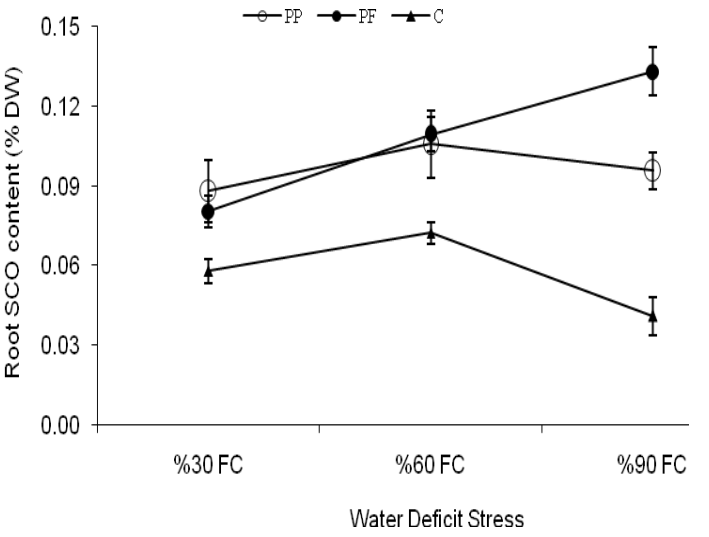

Figure 2- Effects of *Pseudomonas putida*-168 (PP) and *P. fluorescens*-187 (PF) strains on root SCO (scopolamine) content of *Hyoscyamus niger* compared with those of control (C) plants under water deficit stress at 30, 60 and 90 % of FC. Error bars for all data represent standard deviation (±SD, n = 3) based on Least significant difference (LSD) (p < 0.05)

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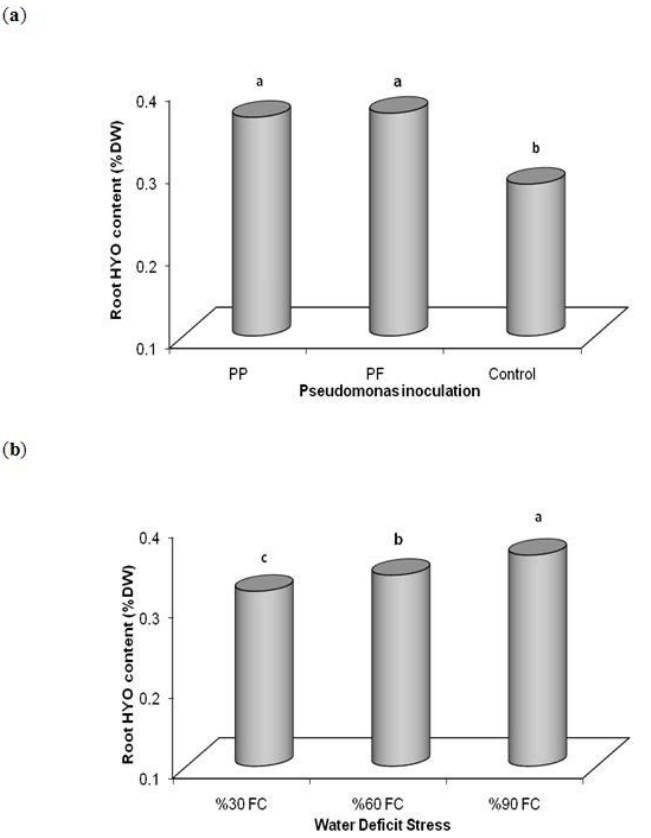

Figure 3- Effects of *Pseudomonas putida*-168 (PP) and *P. fluorescens*-187 (PF) strains on root HYO (hyoscyamine) content of *Hyoscyamus niger* compared with those of control (C) plants (a), effects of water deficit stress at 30, 60 and 90 % of FC levels on root HYO content (b). Mean values in each column with same letter did not differ significantly at p<0.05 according to LSD test

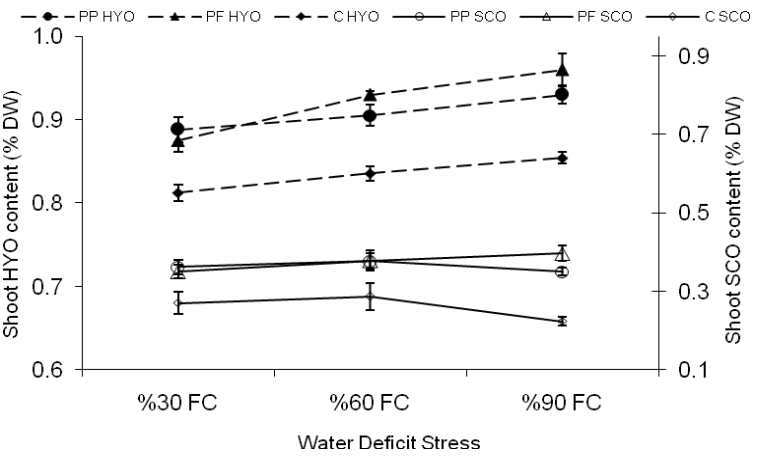

Figure 4- Effects of *Pseudomonas putida*-168 (PP) and *P. fluorescens*-187 (PF) strains on shoot HYO (hyoscyamine, interrupted lines) and SCO (scopolamine, continuous lines) content of *Hyoscyamus niger* compared with those of control (C) plants under water deficit stress at 30, 60 and 90 % of FC. Error bars for all data represent standard deviation (±SD, n = 3) based on Least significant difference (LSD) (p < 0.05)

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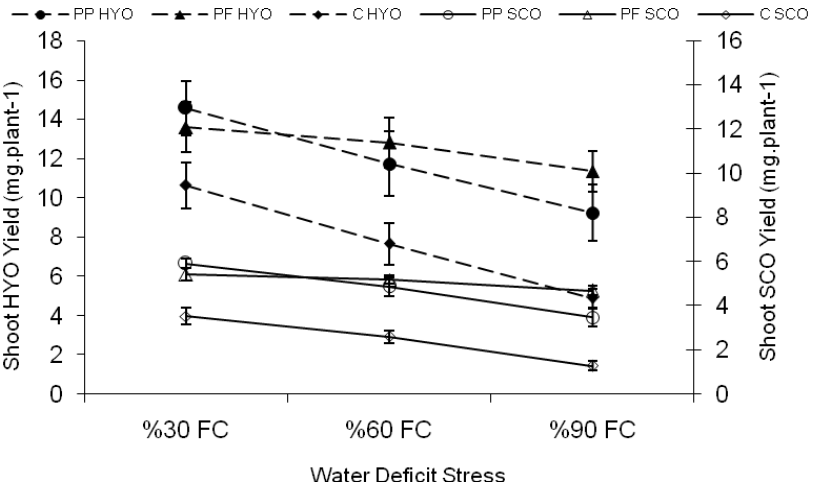

Figure 5- Effects of *Pseudomonas putida*-168 (PP) and *P. fluorescens*-187 (PF) strains on shoot HYO (hyoscyamine, interrupted lines) and SCO (scopolamine, continuous lines) yield of *Hyoscyamus niger* compared with those of control (C) plants under water deficit stress at 30, 60 and 90 % of FC. Error bars for all data represent standard deviation (±SD, n = 3) based on Least significant difference (LSD) test ($p < 0.05$)

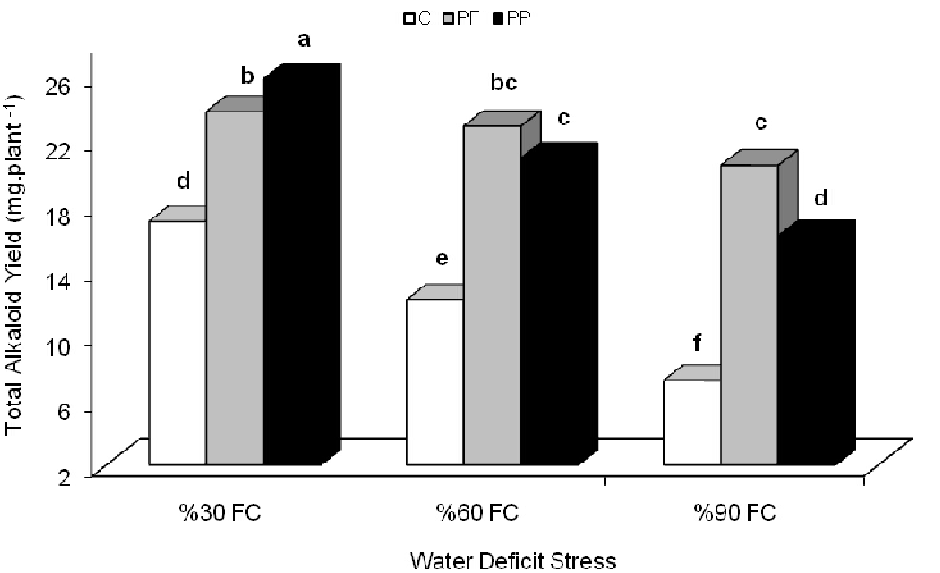

Figure 6- Effects of *Pseudomonas putida*-168 (PP) and *P. fluorescens*-187 (PF) strains on total alkaloids (hyoscyamine+ scopolamine) yield of *Hyoscyamus niger* compared with those of control (C) plants under water deficit stress at 30, 60 and 90 % of FC. Mean values in each column with same letter did not differ significantly at $p<0.05$ according to LSD test

Discussion

Laboratory assays revealed that PF-187 and PP-168 strains were the most effective strains for early seedling development or VI; therefore they were selected for greenhouse experiment. Fluorescent pseudomonads including *putida* and *fluorescens* have substantial effects on plant growing under

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various conditions particularly via auxin secretion [17]. However, production of this phytohormone at the amounts higher than that is needed for plant produces additional levels of ACC (1-aminocyclopropane-1-carboxylic acid), the immediate precursor of ethylene production, which significantly inhibits root elongation and decreases VI and plant growth [18]. Similarly, our results in laboratory experiments showed that the most efficient strains on seedling VI in plate and tube assays were those (PP-168 and PF-187) that produce optimum auxin (Table 1). Fluorescent pseudomonades *(putida* and *fluorescens*) may have considerable effects on tropane alkaloids content and yield under stress conditions by combination of direct and indirect mechanisms [19]. Solubilization of inorganic phosphate, mineralization of organic phosphate, improved nutrient uptake, inhibition of plant ethylene synthesis and enhanced stress resistance are modulated by PGPR as direct mechanisms [20]. In indirect mechanisms, secondary bacterial metabolites are involved such as HCN (hydrogen cyanide) and siderophores production that chelate iron and make it available to the plant root [19]. These traits of PP and PF strains which employed in current study are given in table 2. Rhizosphere and plant dependent mechanisms have been proposed for the stress tolerance mediated by PGPR [21]. Efficiency of PGPR on plant function is determined by their multiple growth-promoting activities especially under stress conditions. Several studies have shown that WDS is one of the major environmental factors which limit the productivity of plants and may cause damage to plant tissues through the formation of radical oxygen species (ROS) [22]. In order to decrease the deleterious effects of ROS, antioxidant promoting systems are required [23] such as PGPR application. Although, WDS is the major limiting factor on crop plants growth and biomass production, enhancement of secondary plant products, solute accumulation and enzyme activities are considered as positive effects of limited water supply in medicinal plants [24]. The use of elicitors is one of the effective means employed to increase the production of important alkaloids [25]. PGPR and osmotic stresses are classified as biotic and abiotic secondary metabolites elicitors in medicinal plants [26, 27]. In our study, rhizobacteria have ability to produce growth regulators such as IAA (Table 1) which could act as elicitor on tropane alkaloid biosynthesis under WDS, resulting in raising alkaloids yield and content. According to our results, PP treated plants under W1 conditions had higher proportion of fine roots compared to other treatments (data not shown) resulting in high HYO and SCO yield in root and shoot. Nakanishi et al. [28] found that root secondary growth is one of the significant causes determining tropane alkaloid composition, which varied based on root diameter, also reported that high levels of littorine (biosynthetic precursor of hyoscyamine), hyoscyamine and 6 β-OH-hyos were observed in fine roots. The plant growth promoting properties of PGPR could be the driving force for alkaloids yield increment in this study. WDS had also positive effects (but less than PGPR) on alkaloids content of plant root and shoot. In current study, shoots showed higher alkaloids accumulation than roots. It is well-established that, root is the location of tropane alkaloid biosynthesis in solanaceous plants, but they may be transported to the aboveground parts of the

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plant [29, 30]. The main reason for the increased production of alkaloids in plants treated with Pseudomonas strains can be compounds that are produced by microorganisms, which act as secondary messenger in secondary metabolites biosynthesis pathway [27]. Bacterial strains used for this study were different with respect to auxin and siderophore production and phosphorous solubilization (Table 2). It was obvious that HYO was the prevalent alkaloid in root and shoot. High total alkaloids yield in low WDS and PGPRs treated plants had a strong positive correlation with biomass production. The highest ratio of SCO to HYO (0.53) and the highest alkaloids (HYO and SCO) content in root and shoot was observed in W3PF treatment.

Conclusion

The results of this study suggest that *H. niger* plants with inoculation of PF under moderate WDS (W2) in addition to have appropriate amounts of each alkaloid content and yield, also have the largest value of SCO, which is the indicator of tropane alkaloids quality. Considering that the aim of commercial production of medicinal plants is to obtain greater amounts of biomass per unit area that also has a higher content of active ingredients (because manufacturing cost of the drug extract should be affordable), it is of a significant importance to have high overall alkaloids yield rather than content for *H. niger* plants since it is not consumed directly as pharmaceutical and the desired active compounds such as HYO and SCO should be extracted from the plant raw materials. Thus, inoculation of *H. niger* roots with PGPR strains especially PF-187, not only alleviated the deleterious stress effects on plant growth to some extent, but also increased elicitation of tropane alkaloids under WDS conditions.

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