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Original Contribution

PGPR STRAINS AFFECT SEEDLING VIGOR INDEX AND SEED SECONDARY METABOLITES ACCUMULATION OF BLACK HENBANE UNDER WATER STRESS

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ABSTRACT
Twenty plant growth promoting rhizobacteria (PGPR) strains belonging to *Pseudomonas putida* (PP) and *P. fluorescens* (PF) with different abilities for auxin production were screened based on their effects on vigor index (VI) of *Hyoscyamus niger* plants. Seedlings radicles and culture media were inoculated with these strains (10^9 CFU/ml). Thereafter, according to the highest values of VI, the effects of two most efficient strains on seed hyoscyamine (HYO) and scopolamine (SCO) accumulation were investigated under three water deficit stress levels as 30 (W1), 60 (W2) and 90 % (W3) water depletion of field capacity from initial of flowering to full pod filling stage. Results showed that different strains of rhizobacteria had variable effects (both negative and positive) on VI in various tested assays. The most efficient strains on VI were those (PP-168 and PF-187) that produce optimum auxin. Seed alkaloids (HYO and SCO) accumulation was significantly affected by water deficit stress and PGPRs inoculation. Alkaloids content was negatively correlated to seed weight. The highest seed alkaloid content (HYO: 0.271%; SCO: 0.135% DW) were achieved under W3 conditions in PF-187 treated plants. By contrast, the maximum seed alkaloid yield (HYO: 1.245; SCO: 0.620 mg.plant^{-1}) were obtained in PP-168 treatment under W1 conditions.

Key words: *Hyoscyamus niger*, vigor index, alkaloids, *Pseudomonas* strains, water deficit stress

INTRODUCTION

Black henbane (*Hyoscyamus niger*) an species in solanaceae family has long been used as a medicinal plant. A cosmopolitan, strong-scented annual or biennial herb, which all it's parts contain tropane alkaloids such as hyoscyamine (HYO) and scopolamine (SCO) (1). These metabolites are used in medicine as anticholinergic, antispasmodic, hypnotic and sedative (2). 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 like *Atropa belladona*, *Datura stramonium* (3).

Although, stress situations negatively affect the growth and development of crops, the contents of secondary metabolites are mostly increased through the positive effects on the metabolic pathways of active compounds synthesis in medicinal plants (4). Plants infection with microorganisms as well as physical factors such as osmotic stresses induced particular secondary metabolite pathways (5). Among the numerous microorganisms in rhizosphere, some have positive effects on plant growth promotion comprising PGPR such as PP and PF strains, which colonize the rhizosphere and roots of

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many plant species and confer beneficial effects including synthesis of phytohormones such as auxins (6), solubilization of minerals, production of siderophores, increases in nutrient uptake and delayed leaf senescence (7). Auxin secreted by Pseudomonas strains, is one of the main reasons to promote plant growth under different conditions including stress (8). It is involved in many growth and behavioral process in the plant life cycle such as root initiation, cell division and enlargement, also it increases root surface area, root elongation and enhances the formation of lateral roots, which result in consequent access to soil nutrients and drought tolerance (9). In recent years, many experiments have been conducted on the role of microbial association like *arbuscular mycorrhizal* symbiosis and plant growth promoting rhizobacteria (PGPR) in drought stressed crop plants (10). However, there have been no comparative studies on effects of *Pseudomonas putida* (PP) and *Pseudomonas fluorescens* (PF) strains under water deficit stress on topane alkaloids yield and content in such a commercially important medicinal plant. Despite many studies on the production of these alkaloids by hairy root cultures, it has not been commercially exploited yet. The objectives of this study were firstly to select two efficient rhizobacteria among twenty PP and PF strains based on their potential for early growth promoting in terms of seedling vigor index under laboratory conditions. Secondly, to investigate the effects of selected strains (as biotic elicitors) on seed weight and seed alkaloids accumulation of *Hyoscyamus niger* plants in combination with water deficit stress (as abiotic elicitor) application under greenhouse situations.

MATERIALS AND METHODS

1. Seed preparation and germination

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 for 10 min and finally rinsed with sterile distilled water. Subsequently, seeds were placed in petri dishes on two layers of filter paper moistened with 4 ml distilled water. After 3 days, 90% of seeds germinated steadily (with 1-2 mm radicle length).

2. Bacterial strains and growth conditions

PGPR strains 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 25ml 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 10^9CFU/ml. The prepared suspensions were used to inoculate henbane radicles and culture media under aseptic conditions.

3. 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%). This experiment was carried out with four replications in a randomized complete block design (RCBD) 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 (11). $VI\ (1) = (RL+SL) \times GP\ (\%)$ and $VI\ (2) = (RDW+SDW) \times GP\ (\%)$. Where 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).

4. 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 (3cm in diameter and 12cm height). Tubes were filled with 120g 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 (12). The experiment was arranged in a randomized complete block design with three replicates for twelve treatments in each genus of *Pseudomonases* strain.

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)
4 Akhgar	2.380
12Akhgar	1.200
73	2.960
174	7.350
99	13.388
169	5.775
173	12.470
187	5.152
196	6.130
CHAO	*

Pseudomonas Putida strains	Mean IAA production(mg/l)
4	8.499
11	6.752
41	47.694
53	30.126
108	9.774
112	31.222
143	32.941
147	29.030
159	76.697
168	17.921

* Variable in different culture media.

5. Water deficit stress induction

Based upon the efficiency of rhizobacteria in enhancing the seedling growth index (*in vitro* and tube assays), two of the most effective PGPR strains namely PP-168 and PF-187 were selected and used in pot trials. The inoculum suspensions were applied evenly within the two holes (1cm depth) on the pot soil surface at a rate of 0.5 ml per seedling using syringe. Thereafter, two inoculated seedlings were immediately sown in each 20 cm × 15 cm pot containing 4 kg soil (both radicle and soil inoculations were carried out). Plants were subjected to water deficit stress from initial of flowering to full pod filling stage (full repining time) for 45 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. Ripening plants with indehiscent capsules, containing seeds turning from grey to brown were harvested, dried indoors at ambient temperature for one week. The seeds weight was determined with a precision of 0.0001 g scale. Then seeds were separated from capsules and finely ground for alkaloid extraction. 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 randomized complete block design (RCBD) was used with factorial treatments of three watering frequencies i.e. 30 (W1), 60 (W2), and 90 % (W3) water depletion of field capacity (FC) and two PGPR strains along with control (without PGPR inoculation) in 3 replications. All pots were kept to the FC up to 45 days after planting.

6. Alkaloid extraction and analysis

Seed samples were grinded into fine powder and sieved with laboratory mesh (size 30, mesh opening 545 μm). A subsample of one gram 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

(13). Alkaloids extracted were identified by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis. Gas 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 (30m×0.25mm×0.25μm). 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 ($mg.plant^{-1}$).

7. Statistical analysis

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 AND DISCUSSION

1. Seedling vigor index and seed dry weight

Analysis of variance from laboratory experiments indicated that different strains of rhizobacteria varied notably in their efficiency for enhancing the weight and length of root, shoot and 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 **(Fig. 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 **(Fig. 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) **(Fig. 1B).** PF-187 strain increased root and shoot elongation by 73% and 51%, respectively compared with uninoculated control (data not shown). In plate assay, PP-159 and PP-168 strains had the highest values of VI **(Fig. 1C)** but in the tube experiment only PP-168 strain was the superior one, which enhanced VI up to 62% over untreated control **(Fig. 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. Laboratory assays revealed that PF-187 and PP-168 strains were the most effective strains for early seedling development; therefore they were selected for greenhouse experiment. Fluorescent pseudomonads including *putida* and *fluorescens* have substantial effects on plant growing under various conditions particularly via auxin secretion (8). However, production of this phytohormone at the amounts higher than that is needed for plant produces additional levels of ACC, the immediate precursor of ethylene production, which significantly inhibits root elongation and decreases VI and plant growth (7). Similarly our results in laboratory experiments showed that the most efficient strains on seedling vigor index (VI) in plate and tube assays were those (PP-168 and PF-187) that produce optimum auxin **(Table 1).** There were significant variations ($p < 0.01$) in seeds of *H. niger* plants dry weight treated with PP-168 and PF-187 when compared with controls **(Table 2).** Generally, the total seed dry weight was significantly decreased by increasing water deficit stress levels, and reduction percentage was very conspicuous in the control plants (27%) compared to PP (8.5%) and PF (4.5%) treated plants. Inoculation with PGPR strains minimized the effects of water stress applied at all levels.

PP-168 strain was found more effective in promoting seed weight, when water stress was applied at W1 condition. Similarly, when water stress was applied at W3 level the maximum increases in seed dry weight were observed because of inoculation with PF-187 strain. *Pseudomonas* strains play an important role in plant growth and physiology by combination of direct and indirect mechanisms (14). Direct mechanisms involve secretion of growth regulators such as auxins, cytokinins and gibberellins, which enhance various stages of plant growth or synthesize enzymes that modulate plant growth and development (9) and also solubilization of inorganic phosphate and mineralization of organic phosphate (15). In our current study, increase of seed dry weight in both PP-168 and PF-187 treated plants compared to control under all water stress treatments could be attributed to these phytohormones, which change the assimilate allocation in plants and affect the growth patterns, which was in agreement with findings of previous studies (16).

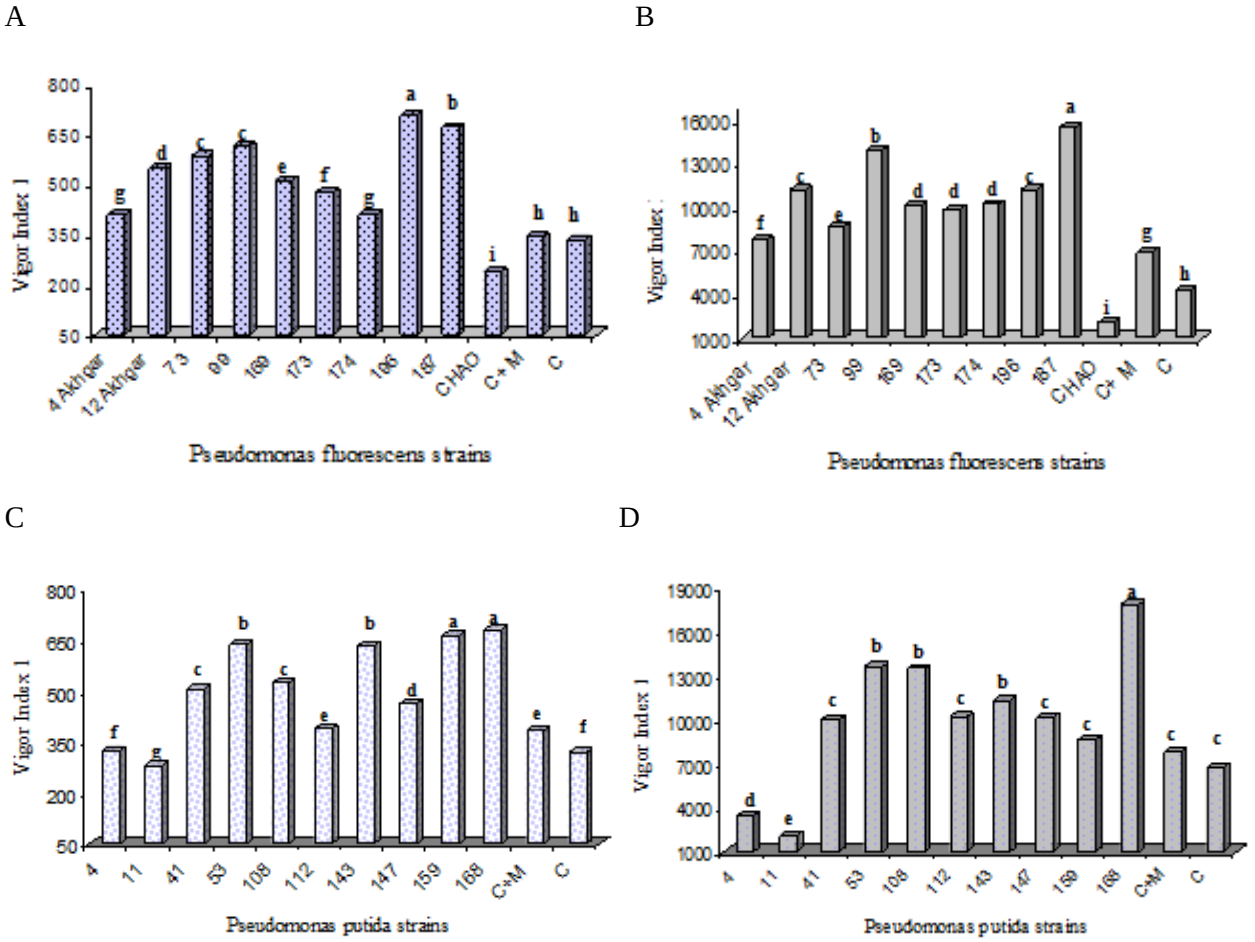

Fig. 1. Effect of *pseudomonas fluorescens* and *Putida* strains inoculation on *hyoscyamus niger* seedling vigor index 1 in plate (A and C) and tube (B and D) assays. C and C+M in horizontal axis refers to control and control plus bacterial medium (TSB) treatment, respectively. Values marked with the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

2. Seed alkaloid accumulation

HYO and SCO content were significantly ($P < 0.05$) influenced by water deficit stress and *Pseudomonas* strains **(Table 2).** The content of seed HYO were increased with increasing water stress in both PGPRs inoculation and untreated control plants (C). The application of PP-168 and PF-187 strains, again separately increased the content of seed HYO, respectively to a significant level. The PGPR strains varied greatly in their effect on the seed SCO content. The greatest accumulation of SCO content was

found in PF-187 treated plants against severe water stress (W3). In contrast, the seed SCO accumulation in PP-168 and in untreated control plants was observed only up to W2 treatment and later it started to decline, particularly in the untreated control plants. The maximum contents of HYO (0.271% DW) and of SCO (0.135% DW) were observed in the PF-187 treated plants under W3 conditions **(Table 2).** Seed alkaloids (HYO and SCO) yield were decreased with increasing water stress in both *Pseudomonas* treated and uninoculated control plants, mainly because of seed dry weight parameter reduction under the water stress conditions. However, the reduction percentage in PP-168 and PF-187 treated plants was lower than uninoculated control. Thus, inoculation with these *Pseudomonas* strains alleviated the deleterious stress effects on seed yield to some extent. Reduction of total seed alkaloids yield (HYO+SCO) in uninoculated control treatments were 3.69 and 13.5- fold less than PP-168 and PF-187 treated plants under severe water stress (W3) treatment, respectively. The highest total seed alkaloids yield were found in the PP-168 treated plants under W1 conditions **(Table 2).** Results showed that PP-168 was the most effective strain under low water stress conditions (W1). Whereas PF-187 had the highest efficiency in improving the seeds alkaloid accumulation of *H.niger* plants in the presence of medium (W2) and severe water stress (W3). These two strains were also characterized for multiple growth promoting attributes **(Table 3).** Microorganisms and osmotic stresses are classified as chemical and physical secondary metabolites elicitors in inmedicinal plants, respectively (17). Rhizobacteria produce growth regulators which act as elicitors on tropane alkaloid biosynthesis resulting in raising alkaloids yield and content. It is of a significant importance to have high overall alkaloids yield rather than content for *H.niger* because 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. The plant growth promoting properties of PGPR could be the driving force for alkaloids yield increment in this study. Water deficit stress had also positive effects (but less than PGPR) on alkaloids content of plant seeds. Increases of free amino acids, soluble proteins and soluble carbohydrates are among the factors contributing to higher alkaloids contents in plants experiencing water stress (18). However, it significantly reduced plant biomass which is key determinant of alkaloid yield per plant. Similar increase of ajmalicine content was observed in *Catharanthus roseus* plants due to PF treatment under drought stress conditions (19). Interaction between elicitors and receptors on the plant cell surface is the main reason of elicitation response and thereby improves the production of secondary metabolites (20). Bacterial strains used for this study were different with respect to auxin and siderophore production and phosphorous solubilization **(Table 3)**. High capacity for organic acid (high P solubility) production could be one of the mechanisms applied by PF-187 to improve the P solubility under water stress and finally enhancing the alkaloid content. The same results have previously been reported for ergot alkaloid production in roots of endophyte-infected tall fescue, which increased directly with phosphorous availability in the soil (21). A close relationship between phosphorous content and water deficit tolerance has been observed earlier (22). Strain PP-168 functioned weakly under W2 and W3 conditions compared to PF-187. Biochemical test have shown that PP-168 was a proficient strain for HCN production. Although, HCN does not participate in growth, is a phytotoxic agent capable of inhibiting enzymes involved in major metabolic processes (23). HCN production may be one the potential mechanisms linked with lower efficiency of PP-168 in *H. niger* plants exposed to moderate and high water stress conditions. Many of *Pseudomonas* strains have been found to produce the secondary metabolites such as salicylic acid (24), which promotes the plant growth and increases the plant immunity by activating defense systems (25). Ajungla et al. (26) found that salicylic acid can also act as abiotic elicitors stimulating the production of HYO and SCO in the *in vitro* root culture of *Datura metel*. A significant ($p < 0.05$) linear relationship between total seed dry weight and their alkaloids (HYO and SCO) yield was observed in *H. niger* plants under different water stress levels and *Pseudomonas* strains. It was obvious that HYO was the prevalent alkaloid in seeds. Increase of alkaloids content under water stress conditions is associated with a reduction of seed dry matter production. According to our

results, the highest ratio of SCO to HYO (0.5) and the highest total alkaloids (HYO + SCO) was achieved by application of PP-168 strain in combination with 30% water depletion from field capacity.

Table 2. *Mean values for seed weight, seed alkaloids (HYO and SCO) content and yield (mean ± S.D., n=3) of H. niger inoculated with Pseudomonas strains at different water deficit stress levels.*

Treatment	Seeds weight (g. plant^{-1})	Seed alkalid content (%DW)		Seed alkalid yield (mg.plant^{-1})		Total alkaloid yield (mg.plant^{-1})
		HYO	SCO	HYO	SCO	
W1PP	5.212±0.24	0.239±0.004	0.119±0.006	1.245±0.113	0.620±0.045	1.865±0.143
W1PF	4.782±0.17	0.226±0.003	0.112±0.004	1.080±0.103	0.535±0.041	1.615±0.131
W1C	4.163±0.22	0.177±0.007	0.075±0.012	0.736±0.145	0.312±0.082	1.048±0.224
W2PP	4.934±0.23	0.251±0.004	0.124±0.003	1.238±0.097	0.611±0.033	1.849±0.122
W2PF	3.956±0.28	0.258±0.009	0.128±0.006	1.020±0.114	0.506±0.034	1.526±0.126
W2C	2.431±0.16	0.182±0.012	0.081±0.014	0.442±0.078	0.196±0.064	0.638±0.128
W3PP	2.843±0.24	0.262±0.006	0.113±0.003	0.744±0.057	0.321±0.032	1.065±0.071
W3PF	3.291±0.15	0.271±0.008	0.135±0.007	0.891±0.094	0.444±0.041	1.335±0.138
W3C	1.124±0.31	0.201±0.009	0.047±0.03	0.225±0.055	0.052±0.033	0.277±0.108
F water(w)	80.45**	15.25**	117.84**	107.03**	15.25**	70.20**
F pseudomonas(p)	2860.51**	582.53**	91.46**	1494.29**	582.53**	1307.96**
*F w*p*	5.05**	4.06*	3.10*	11.09**	4.06*	9.37**

W1, W2 and W3: water deficit stress at 30, 60 and 90 % of water depletion from field capacity, respectively. PP, PF and C: *P. putida*-168, *P. fluorescens*-187 strains and Control, respectively.*P < 0.05; **P < 0.01.

Table 3. *Multiple plant growth promoting characteristics of Pseudomonas putida-168 (PP) and Pseudomonas fluorescens-187 (PF) strains.*

Pseudomonas strains	PGPR characteristics				Ecological site of strains isolation (rhizosphere)
	Phosphorus solubility	Siderophore production	HCN production	ACC deaminase activity	
PP- 168	-	2.25	high	-	Wheat (cv. Gaskozhen*)
PF-187	+	1.25	-	-	Wheat (cv. Azar**)

PGPR: Plant Growth Promoting Rhizobacteria, P (phosphorus), *Irrigated variety (Hydrophyl), **Dry land variety (Xerophyl), HCN: Hydrogen Cyanide, ACC: 1-aminocyclopropane-1-carboxylic acid.

CONCLUSION

It can be concluded that different PP and PF strains varied extremely in their efficiency for promotion of seedling early growth and VI due to their various abilities on auxin production and other multiple plant growth promoting characteristics. Among all tested PGPR strains PP-168 and PF- 187 were two most effective

strains under *in vitro* and growth room conditions. Inoculation of *H.niger* plants by PP-168 and PF-187 remarkably improved the seeds alkaloids content and yield. Water stress only improved the content of these alkaloids, but decreased seed dry weight resulting in low alkaloids yield. Our results showed that PP-168 strain had the highest seed alkaloids yield under low water stress conditions whereas PF-187 was the effective strain under moderate and severe water stress situations. Therefore, application of suitable PGPR strains should be based on multiple plant growth promoting characteristics and their ecological adaptation with respective abiotic stress of the host plant. Integrative use of effective PGPR strains (biotic elicitor) and a physical elicitor such as water stress sounds to be an encouraging new method and eco-friendly strategy for increasing tropane alkaloids yield in *H. niger* plants.

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