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Role of plant growth promoting rhizobacteria on antioxidant enzyme activities and tropane alkaloid production of *Hyoscyamus niger* under water deficit stress

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Abstract: This study examined the effects of inoculation with 2 rhizobacteria strains, *Pseudomonas putida* (PP) and *Pseudomonas fluorescens* (PF), on growth parameters, chlorophyll, proline, leaf relative water content (RWC), antioxidant enzyme activities (including superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT)), tropane alkaloids (such as hyoscyamine (HYO) and scopolamine (SCO)), and production of *Hyoscyamus niger* under 3 water deficit stress (WDS) levels, i.e. 30% (W1), 60% (W2), and 90% (W3) water depletion of field capacity. The results showed that inoculation with PP and PF strains minimized the deleterious effects of WDS on growth parameters. The activities of SOD and POX in root and leaf were increased to a significant extent with inoculation of PP and PF, and also with WDS treatment, whereas CAT activity decreased with increasing WDS, except for in plants treated with the PF strain. The maximum proline, HYO, and SCO content were recorded in PF-treated plants under W3 conditions. In contrast, the highest root and shoot alkaloids yield were obtained in plants bacterized with PP against W1 conditions. PP was the most effective strain under low WDS, PF had the highest efficiency in improving the growth and alkaloid production in the presence of severe (W3) WDS. Integrative use of effective plant growth promoting rhizobacteria (PGPR) and WDS could be an encouraging and eco-friendly strategy for increasing alkaloid yield and content in *Hyoscyamus niger* organs.

Key words: *Hyoscyamus niger*, rhizobacteria, water deficit stress, tropane alkaloids, antioxidant enzymes

1. Introduction

Black henbane (*Hyoscyamus niger*) is an important medicinal plant of the family Solanaceae. It is a rich source of tropane alkaloids such as hyoscyamine (HYO) and scopolamine (SCO), which are generally used in medicine as anticholinergic, antispasmodic, mildly pain-relieving, hypnotic, hallucinogenic, pupil-dilating, and sedative compounds (1). Due to its fewer side effects on the nervous system, SCO is preferred and has higher commercial demand (2). Because of the complex chemical structures of these alkaloids, industrial synthesis has been found to be prohibitively expensive; therefore they are mainly obtained from plant resources of the family Solanaceae like *Atropa belladona* or *Datura stramonium* (3). These metabolites have also been reported in other plant families, e.g., *Orchidaceae*, *Euphorbiaceae*, *Cruciferae*, and *Convolvulaceae*, and in the fungus *Amanita muscaria* (4). A plant's fine roots without secondary growth have been found to be the principal site of tropane alkaloid production, where the main enzymes of their biosynthetic pathway are localized (5).

Infection with microorganisms, as well as physical factors such as osmotic stresses, induce particular secondary metabolite pathways (6). Among the numerous microorganisms in the rhizosphere, some have positive effects on plant growth promotion. These microorganisms are plant growth promoting rhizobacteria (PGPR) such as *Pseudomonas putida* (PP) and *Pseudomonas fluorescens* (PF), which colonize the rhizosphere and roots of many plant species and confer beneficial effects to plants. Although drought stress negatively affects 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. This type of abiotic stress affects the plant water status at the cellular and whole plant level, causing specific as well as unspecific reactions and damage (7).

Environmental stresses impair the electron transport system, leading to the generation of reactive oxygen species (ROS) such as H_2O_2, O^{-2}, and OH^- that cause rapid cell damage (8). These compounds initiate damage

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in the chloroplast and cause a cascade of damaging effects, including chlorophyll destruction. Moreover, root meristem activity is very sensitive to ROS; these compounds were accumulated in the differentiation zone, where root hairs grow (9). In plants the metabolism of ROS is kept in dynamic balance. Under water deficit stress (WDS), the balance is upset and antioxidant systems such as superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT), along with the antioxidant ascorbic acid and glutathione, scavenge the reactive free radicals (10). Inoculation of plants with native suitable microorganisms may decrease the deleterious effects of environmental stresses and increase stress tolerance of plants by a variety of mechanisms, including synthesis of phytohormones such as auxins, solubilization of minerals like phosphorus, production of siderophores, and increases in nutrient uptake, leaf area, chlorophyll, and soluble leaf protein content (11). In recent years, many experiments have been conducted on the role of microbial association like *Arbuscular mycorrhizal* symbiosis and PGPR in drought-stressed crop plants (12). However, there have been no comparative studies about the role of PP and PF strains under WDS on antioxidant enzyme activities or secondary metabolite production (especially tropane alkaloids) in such a commercially important medicinal plant. A review of the literature revealed that in most of the studies no investigations have been performed regarding alkaloid yield and the content of these metabolites on a whole plant basis under respective treatments. The objective of the present study was to investigate the growth promoting effects of 2 promising rhizobacteria strains, *Pseudomonas putida* and *Pseudomonas fluorescens*, as biotic elicitors in combination with WDS as abiotic elicitor, on *Hyoscyamus niger* root and shoot growth, antioxidant enzymes (SOD, POX,CAT) activity, and the production of 2 main tropane alkaloids, hyoscyamine and scopolamine.

2. Materials and methods

2.1. Seed preparation and germination

Seeds of *Hyoscyamus niger* were provided by the Agricultural and Natural Resources Center in Esfahan, Iran. Black henbane seeds generally have a low germination rate under normal laboratory conditions. Therefore, seeds were treated with 250 mg L^{-1} 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, 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 2 layers of filter paper (Whatman no. 1) moistened with 4 mL of distilled water. After 3 days, 90% of seeds germinated steadily (with 1–2 mm radicle length).

2.2. Bacterial strains and inoculation

Based upon the efficiency of rhizobacteria in enhancing the seedling growth index, whose results are in separate preliminary in vitro and sand culture assays, 2 of the most effective PGPR strains with different multiple plant growth promoting activities, namely PP-168 and PF-187 (shortened to PP and PF, respectively), from 20 strains were selected and used in this study (Table 1). To prepare inocula, a single colony of each PGPR strain was transferred to 100-mL flasks containing 25 mL of tryptone soybean broth (TSB) and grown aerobically in the 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^{-1}. The prepared suspensions were used to inoculate henbane radicles and culture media under aseptic conditions.

2.3. Plant growth conditions and water deficit stress induction

The inoculum suspensions were applied on both seedlings' radicles (1–2 mm length) and evenly within the 2 holes (1 cm depth) on the pot soil surface at a rate of 0.5 mL per seedling using a syringe. Thereafter, 2 inoculated seedlings were immediately transplanted to a plastic pot (25 cm diameter and 30 cm deep) containing 8 kg of soil, which had been sterilized by autoclaving at 105 °C for 60 min over 3 consecutive days. Untreated radicles and culture media served as controls (C). Sterilized broth (free of bacterial population) was applied in the case of

Table 1. 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 (μg mL^{-1})	Siderophore production	IAA production* (mg L^{-1})	HCN production score	ACC deaminase activity	
PP-168	-	2.25	17.921	5 (high)	+	Wheat (cv. Gaskozhen**)
PF-187	438.38	1.25	5.152	-	-	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, ACC: 1-aminocyclopropane-1-carboxylic acid. + and -: with and without activity, respectively.

the control plants. The experiment was conducted in a greenhouse located at the Faculty of Agricultural Science at Tehran University (Karaj, Iran). During the experiment, the temperature ranged from 26 °C to 30 °C and relative humidity was between 65% and 75%. Plants were subjected to WDS treatments for 60 days, starting 45 days after planting. The field capacity (FC) of soil was determined to be 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 (during morning hours). Monitoring of the soil moisture levels was conducted using a gravimetric method. 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 a randomized complete block design (RCBD) with factorial experiments of 3 water deficit stress (WDS) levels at 30% (W1), 60% (W2), and 90% (W3) water depletion of field capacity and 2 PGPR strains along with controls (without PGPR inoculation) in 3 replications. All the pots were kept to the field capacity up to 45 days after planting.

2.4. Plant growth measurements and analysis

After 60 days of WDS, all pots were harvested at the full flowering stage. Plants were uprooted, washed carefully, and separated into fine roots (<1 mm diameter), coarse roots (≥1 mm diameter), leaves, and stems for analysis. Leaves were counted and then total leaf area per plant was measured using a LICOR Photoelectric Area Meter. The plant materials were then shade dried, weighed with a precision (0.0001 g) scale, finely powdered in an electronic blender, and kept in separate containers for tropane alkaloid extraction.

2.5. Leaf relative water content (RWC)

RWC was measured to evaluate plant water status on 3 leaves collected from each of 2 plants at 15, 30, 45, and 60 days after stress (DAS). The 4th fully expanded leaf (from the top) was weighed immediately after cutting (FW), hydrated to full turgidity by floating in distilled water, kept at room temperature (22 °C) for 24 h to obtain turgid weight (TW), and finally oven dried for 48 h at 70 °C to measure dry weight (DW). RWC was calculated by the following formula as described by Jeon et al. (13): RWC = [(FW – DW) / (TW – DW)] × 100.

2.6. Chlorophyll pigment

Chlorophyll was extracted from the 4th fully expanded leaf from the top at the end of the experimental period. To extract chlorophyll, 50 mg of fresh leaf was homogenized in 5 mL of acetone (80% V/V). The extract was read at 645 nm (chlorophyll *a*) and 663 nm (chlorophyll *b*) in a UV-160 a spectrophotometer and chlorophyll contents were calculated using the equations suggested by Lichtenthaler (14):

$$\text{Chl } a \text{ (mg g}^{-1}\text{ FW)} = 11.75 \times A_{663} - 2.35 \times A_{645}$$

$$\text{Chl } b \text{ (mg g}^{-1}\text{ FW)} = 18.61 \times A_{645} - 3.96 \times A_{663}$$

The same leaf that was used to measure RWC was first used to measure leaf greenness using the Soil Plant Analysis Development (SPAD) chlorophyll meter (Minolta SPAD-502 chlorophyll meter, Tokyo, Japan).

2.7. Proline content

The proline content was quantified by the acid-ninhydrin procedure of Bates et al. (15). The leaf tissues (0.5 g) were ground with 3% sulfosalicylic acid (10 mL) and clarified by centrifugation. Supernatant (2 mL) was mixed with the same volume of acid-ninhydrin and acetic acid, the mixture was oven-incubated at 100 °C for 1 h, and the reaction was finished in an ice bath. The reaction mixture was extracted with 4 mL of toluene using a vortex mixer for 15–20 s and absorbance was read at 520 nm.

2.8. Antioxidant enzyme assays

A crude enzyme extract was prepared by homogenizing 0.5 g of frozen root and leaf tissues in an extraction buffer containing 0.5% Triton X-100 and 1% polyvinyl pyrrolidone in 100 mM potassium phosphate buffer (pH 7.0) using a chilled mortar and pestle. The homogenate was centrifuged and the supernatant was used for the following enzyme assays.

2.8.1. Superoxide dismutase (SOD, EC 1.15.1.1) activity

Total SOD activity was determined according to Beauchamp and Fridovich (16). The reaction mixture contained 1.17×10^{-6} mol L^{-1} riboflavin, 0.1 mol L^{-1} methionine, 2×10^{-5} mol L^{-1} KCN, and 5.6×10^{-5} mol L^{-1} nitroblue tetrazolium (NBT) salt dissolved in 3 mL of 0.05 mol L^{-1} sodium phosphate buffer (pH 7.8). Three milliliters of the reaction medium was added to 1 mL of enzyme extract. The mixtures were illuminated in glass test tubes by 2 sets of Philips 40-W fluorescent tubes in a single row. The absorbance was read at 560 nm in the spectrophotometer against the blank. SOD activity is expressed in U mg^{-1} protein (U = change in 0.1 absorbance h^{-1} mg^{-1} protein under assay conditions).

2.8.2. Catalase (CAT, EC 1.11.1.6) activity

Total CAT activity was assayed according to the method of Chandlee and Scandalios (17). The assay mixture contained 2.6 mL of 50 mmol L^{-1} potassium phosphate buffer (pH 7.0), 0.4 mL of 15 mmol L^{-1} H_2O_2, and 0.04 mL of enzyme extract. Changes in absorbance were read at 240 nm. The enzyme activity was expressed in U mg^{-1} protein (U = 1 mM of H_2O_2 reduction min^{-1} mg^{-1} protein). The enzyme protein was estimated by the method of Bradford (18) for all the enzymes.

2.8.3. Peroxidase (POX, EC 1.11.1.7) activity

Total POX activity was determined by the method of Kumar and Khan (19). The assay mixture for POX contained 2 mL of 0.1 mol L^{-1} phosphate buffer (pH 6.8), 1 mL of 0.01 mol

L^{-1} pyrogallol, 1 mL of 0.005 mol L^{-1} H_2O_2, and 0.5 mL of enzyme extract. The solution was incubated for 5 min at 25 °C, after which the reaction was terminated by adding 1 mL of 2.5 mol L^{-1} H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5 mol L^{-1} H_2SO_4 at zero time. The activity was expressed in U mg^{-1} protein, with 1 U defined as the change in the absorbance of 0.1 min^{-1} mg^{-1} protein.

2.9. Alkaloid extraction

Root and shoot samples were air dried, ground into fine powder, and sieved with a laboratory mesh (size 30, mesh opening 545 μm). A subsample of 2 g from each sample was added to an appropriate volume of $CHCl_3$:MeOH:NH_4OH 25%, (15:5:1), sonicated for 20 min, and then kept in a water bath (40 °C) for 1 h. The subsequent sample preparation and alkaloids extraction were based essentially on the method described by Kamada (20).

2.10. Alkaloid analysis and quantification

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 3 °C min^{-1}, and maintained for 3 min. The flow rate of helium was 0.8 mL min^{-1}. Then 1 μL of extract was directly injected into the gas chromatograph. Each extraction was replicated 3 times and the compound percentages are the means of the 3 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 50 °C to 285 °C at 3 °C min^{-1}, and helium was used as carrier gas (0.8 mL min^{-1}). 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 standard substances (HYO. HCl and SCO. HBr, Merck). The total tropane alkaloid (HYO + SCO) yield was quantified by both alkaloid content and biomass production: Total alkaloid yield (mg $plant^{-1}$) = Alkaloid content (% DW) × Plant dry weight (mg $plant^{-1}$).

2.11. Statistical analysis

All analyses were performed based on a randomized complete block design (RCBD) with factorial experiments. The data were subjected to ANOVA and were analyzed using SAS version 9.1 (CoHort Software). Probabilities of significance were used to test for significance among treatments and interactions, and lowest standard deviations (LSD) tests were used to compare means ($P < 0.05$). Values obtained were expressed as mean ± standard deviation from 3 replications (n = 3) of each treatment.

3. Results

3.1. Leaf parameters and growth variables

The results indicated that there were significant ($P < 0.01$) interactions between WDS and the 2 *Pseudomonas* strains compared with untreated controls for most of the investigated traits. WDS resulted in a serious reduction in *H. niger* growth. Leaf numbers decreased with the increase in water stress levels, but PP- and PF-treated plants had lower reduction percentages compared to untreated control plants (C). Under low WDS (W1) conditions, the highest leaf number was recorded in PP-treated plants, followed by PF, and the lowest leaf number was recorded in the control treatment (C). At moderate (W2) and severe water stress (W3) levels, PP and PF strains caused the same response, but PF-treated plants had a major advantage in terms of leaf numbers in W3 conditions (Table 2). Leaf area variations had a similar trend as leaf number variations. Leaf greenness values were significantly decreased in untreated plants exposed to WDS. Plant leaves in the W3PF treatment had the highest SPAD chlorophyll meter readings at the end of the experiment.

3.2. Proline

The PGPR strains varied greatly in their effect on proline content. The greatest accumulation of proline was found in PF-treated plants against severe WDS. In contrast, proline accumulation in PP-treated plants and in untreated control plants was observed only up to the W2 treatment level and it later started to decline, particularly in the untreated control plants (Table 2).

3.3. Chlorophyll

Chlorophyll *a*, *b*, and total (*a* + *b*) chlorophyll contents dropped in untreated plants under severe WDS, but the ratio between chlorophyll *a* and *b* (*a*/*b*) had an upward trend with increasing water stress. The results also showed that chlorophyll *b* was more sensitive to WDS than chlorophyll *a*. Inoculation with *Pseudomonas* strains significantly improved chlorophyll contents. In both strain treatments, the content of chlorophyll *a* and *b* increased in response to water stress ($P < 0.01$), but the ratio of chlorophyll *a* and *b* significantly decreased with increasing WDS (Table 2).

3.4. Relative water content (RWC)

Leaf RWC was significantly ($P < 0.01$) higher in the plants experiencing PP and PF strains under all WDS conditions than their respective controls. RWC at 15 DAS was fairly high in most of the treatments. W1PP and W3C had the highest (92.1%) and lowest (79.07%) values, respectively. RWC for untreated control plants subjected to water stress

Table 2. Mean values for leaf number, total leaf area, leaf greenness, proline, and chlorophyll content (mean ± SD, n = 3) of *H. niger* inoculated with *Pseudomonas* strains at different water deficit stress levels.

Treatment	Leaf number (no. plant^{-1})	Leaf area (cm^2 plant^{-1})	Leaf greenness	Proline (mg g^{-1} DW)	Chlorophyll (mg g^{-1} FW)			
					a	b	a + b	a/b
W1PP	27.2 ± 0.71	354.1 ± 7.94	53.60 ± 1.02	0.27 ± 0.021	0.058 ± 0.001	0.102 ± 0.003	0.160 ± 0.004	0.57 ± 0.007
W1PF	24.3 ± 0.63	347.3 ± 6.43	51.55 ± 1.32	0.24 ± 0.013	0.056 ± 0.001	0.097 ± 0.002	0.153 ± 0.003	0.57 ± 0.012
W1C	22. 3 ± 0.61	301.7 ± 5.60	46.33 ± 2.19	0.19 ± 0.022	0.050 ± 0.003	0.091 ± 0.002	0.142 ± 0.003	0.55 ± 0.040
W2PP	21.2 ± 0.54	315.6 ± 7.56	56.43 ± 1.17	0.36 ± 0.014	0.062 ± 0.001	0.112 ± 0.004	0.174 ± 0.005	0.56 ± 0.006
W2PF	22.6 ± 0.77	329.3 ± 13.21	60.19 ± 1.26	0.39 ± 0.035	0.064 ± 0.003	0.116 ± 0.001	0.181 ± 0.003	0.55 ± 0.020
W2C	14.01 ± 0.68	256 ± 7.42	41.42 ± 1.59	0.25 ± 0.016	0.053 ± 0.002	0.080 ± .0003	0.113 ± 0.001	0.66 ± 0.050
W3PP	17.22 ± 1.32	273.1 ± 7.16	59.75 ± 1.21	0.34 ± 0.021	0.067 ± 0.002	0.122 ± 0.004	0.189 ± 0.007	0.54 ± 0.004
W3PF	19.50 ± 1.19	298.4 ± 10.2	63.17 ± 2.42	0.48 ± 0.031	0.076 ± 0.003	0.139 ± 0.0005	0.214 ± 0.003	0.55 ± 0.020
W3C	10.7 ± 0.63	179.1 ± 9.81	37.04 ± 1.37	0.14 ± 0.002	0.032 ± 0.003	0.045 ± 0.004	0.077 ± 0.006	0.71 ± 0.050
F WDS × PGPR	8.18**	36.06**	34.87**	7.88*	189.52**	159.43**	229.65**	14.26**

W1, W2, and W3: water deficit stress (WDS) at 30%, 60%, and 90% of water depletion from field capacity, respectively. PP, PF, and C: Pseudomonas fluorescens strain 187, *P. putida* strain 168, and control, respectively. *P < 0.05; **P < 0.01.

(W2C and W3C) started to decline at 30 DAS, sharply declined at 45 DAS, and then dropped to a very low value (57%) during the final drought stress period. PP- and PF-treated plants under the highest water supply (W1PP and W1PF) were found to have RWC in the range of 91%–92% (initial values) and showed a smaller decrease during the WDS period, i.e. it decreased gradually within a narrow range (Figure).

3.5. Root and shoot biomass

There were significant variations (P < 0.01) in shoot (leaf and stem) dry weight of *H. niger* plants treated with PP and PF when compared with the controls (Table 3). Generally, the total shoot dry weight was significantly

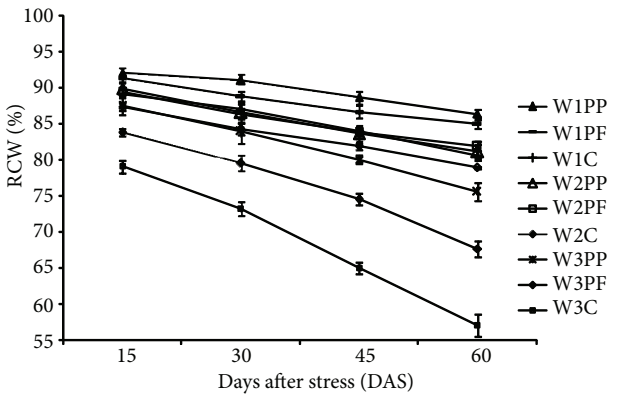

Figure. Leaf relative water content (RWC) variations of *Hyoscyamus niger* with inoculation of *Pseudomonas putida*-168 (PP) and *P. fluorescens*-187 (PF) strains compared with those of control (C) plants under water deficit stress levels; W1, W2, and W3 (30%, 60%, and 90% water depletion of field capacity, respectively) at 15, 30, 45, and 60 days after stress (DAS). Error bars for all data represent standard deviation (±SD, n = 3) based on least significant difference (LSD) (P < 0.05).

decreased by increasing WDS levels. The reduction ratio was very conspicuous in the control plants (2.2-fold reduction) compared to PP- and PF-treated plants (1.6- and 1.3-fold reduction, respectively). The PP strain was more effective in promoting shoot weight when WDS was applied at the W1 level. Similarly, when water stress was applied at the W3 level, the maximum increases in shoot dry weight were observed in plants inoculated with the PF strain. Under W3 conditions, leaf number (Table 2) was 82.2% higher in PF-inoculated plants than in the controls, which is reflected in a 2.06-fold increase in shoot weight. WDS also led to a reduction in root mass as well as to a reduction in shoot dry weight, but increased the root to shoot ratio. Severe WDS (W3) was most intense for fine roots, i.e. roots less than 1 mm in diameter were affected more severely (3.2-fold reduction) than coarse roots (2.4-fold reduction) in the uninoculated control plants compared to W1 conditions. Inoculation with these strains alleviated to some extent the effects of WDS on fine root growth compared to the control plants (Table 3).

3.6. Antioxidant enzymes

There was a significant interaction between SOD, POX, and CAT activities in the roots and leaves of *H. niger* plants under PGPR inoculation and WDS treatments. Different parts of the plant under different treatments showed varied enzyme activity (Table 4). The antioxidant enzyme SOD activity in root and leaf organs increased with increasing WDS in plants inoculated with both PP and PF strains. In the case of control plants, SOD activity increased with increasing WDS up to moderate water stress. The highest level of WDS decreased the specific SOD activity, as shown in Table 4. The highest SOD activity (23.821 U mg^{-1} protein) was recorded in a W3PF-treated leaf sample

Table 3. Biomass production (mean ± SD, n = 3) of *H. niger* inoculated with *Pseudomonas* strains at different water deficit stress levels.

Treatment	Shoot dry weight (g plant^{-1})			Root dry weight (g plant^{-1})		
	Leaf	Stem	Total	Fine root	Coarse root	Total
W1PP	9.03 ± 0.114	7.37 ± 0.34	16.40 ± 0.13	7.21 ± 0.13	4.68 ± 0.18	11.89 ± 0.35
W1PF	8.60 ± 0.080	6.93 ± 0.66	15.53 ± 0.12	6.44 ± 0.17	4.89 ± 0.11	11.33 ± 0.26
W1C	6.73 ± 0.141	6.37 ± 0.34	13.10 ± 0.13	4.80 ± 0.248	3.65 ± 0.32	8.45 ± 0.55
W2PP	7.27 ± 0.107	5.70 ± 0.26	12.97 ± 0.12	5.20 ± 0.11	3.82 ± 0.16	9.02 ± 0.26
W2PF	7.67 ± 0.046	6.13 ± 0.36	13.80 ± 0.65	5.60 ± 0.13	4.39 ± 0.11	9.99 ± 0.20
W2C	4.80 ± 0.048	4.37 ± 0.12	9.17 ± 0.11	2.95 ± 0.055	2.27 ± 0.21	5.22 ± 0.28
W3PP	5.53 ± 0.121	4.40 ± 0.12	9.93 ± 0.23	3.99 ± 0.092	3.01 ± 0.091	7.00 ± 0.19
W3PF	6.40 ± 0.093	5.43 ± 0.12	11.83 ± 0.16	4.95 ± 0.18	3.12 ± 0.135	8.07 ± 0.24
W3C	2.90 ± 0.076	2.83 ± 0.14	5.73 ± 0.15	1.46 ± 0.045	1.51 ± 0.18	2.97 ± 0.17
F WDS × PGPR	21.40**	3.10*	13.68**	10.61**	5.40**	8.90**

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

and the lowest activity (6.97 U mg^{-1} protein) was recorded in the W1C root sample. The CAT activity in root and leaf samples increased with increasing WDS only in PF-treated plants, but plants treated with the PP strain and control uninoculated plants showed reduced activity of this enzyme under WDS conditions. A gradual increase was observed in leaf CAT activity under 60% FC with PP-treated (W2PP) plants in comparison with the controls. Increasing the WDS caused a marked increase in total POX activity of both roots and leaves in PGPR-treated and control plants, particularly in the PF-treated plants under severe WDS. Among all antioxidant activities, the maximum reduction was observed in CAT activity in the leaves of untreated control plants under severe WDS compared to W1 (Table 4).

3.7. Tropane alkaloid content and yield

In *Pseudomonas* strain-treated and uninoculated control plants, the SCO content of roots increased significantly with increasing WDS up to W2 treatment and later started to decline, except for PF-treated plants, which kept a continuously upward trend (Table 5). The largest root SCO content was observed in the PF-treated plants under W3 conditions. There was no significant interaction among the treatments in terms of root HYO content, but it was influenced by PGPR and WDS separately. The maximum root HYO content was seen in the W3 treatment, while

Table 4. Superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) activities of roots and leaves in *H. niger* plants inoculated with *Pseudomonas* strains at different water deficit stress levels (mean ± SD, n = 3).

Treatment	Antioxidant enzyme activity (U mg^{-1} protein)					
	Root enzymes			Leaf enzymes		
	SOD	CAT	POX	SOD	CAT	POX
W1PP	7.45 ± 0.086	2.183 ± 0.074	3.630 ± 0.192	12.887 ± 0.149	2.861 ± 0.088	5.507 ± 0.281
W1PF	7.22 ± 0.041	1.983 ± 0.090	2.587 ± 0.134	11.304 ± 0.056	2.967 ± 0.095	4.40 ± 0.341
W1C	6.97 ± 0.034	1.036 ± 0.045	2.065 ± 0.082	8.954 ± 0.244	2.550 ± 0.012	2.113 ± 0.121
W2PP	9.29 ± 0.112	1.250 ± 0.237	5.493 ± 0.144	14.298 ± 0.132	3.119 ± 0.028	7.050 ± 0.107
W2PF	10.33 ± 0.186	2.762 ± 0.157	4.825 ± 0.041	17.375 ± 0.053	3.217 ± 0.086	6.40 ± 0.182
W2C	8.00 ± 0.047	0.830 ± 0.098	2.435 ± 0.040	11.019 ± 0.135	0.933 ± 0.145	2.207 ± 0.105
W3PP	13.89 ± 0.124	0.945 ± 0.132	6.729 ± 0.034	19.615 ± 0.067	1.094 ± 0.078	9.37 ± 0.163
W3PF	15.02 ± 0.145	3.784 ± 0.141	8.354 ± 0.229	23.821 ± 0.201	3.337 ± 0.097	13.12 ± 0.041
W3C	7.27 ± 0.196	0.465 ± 0.056	2.478 ± 0.098	9.849 ± 0.223	0.2 10 ± 0.057	2.735 ± 0.101
F WDS × PGPR	13.74**	21.57*	16.38**	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: *Pseudomonas fluorescens* strain 187, *P. putida* strain 168, and control, respectively. *P < 0.05; **P < 0.01.

Table 5. Root and shoot alkaloids (HYO and SCO) content (mean ± SD, n = 3) of *H. niger* inoculated with *Pseudomonas* strains at different water deficit stress levels.

Treatment	Root alkaloid content (%DW)		Shoot alkaloid content (%DW)	
	HYO	SCO	HYO	SCO
W1PP	0.351 ± 0.005	0.088 ± 0.005	0.888 ± 0.006	0.362 ± 0.005
W1PF	0.334 ± 0.005	0.080 ± 0.004	0.875 ± 0.006	0.350 ± 0.007
W1C	0.270 ± 0.008	0.058 ± 0.012	0.812 ± 0.009	0.270 ± 0.01
W2PP	0.363 ± 0.005	0.106 ± 0.002	0.905 ± 0.005	0.377 ± 0.004
W2PF	0.369 ± 0.008	0.109 ± 0.006	0.930 ± 0.005	0.378 ± 0.004
W2C	0.283 ± 0.01	0.072 ± 0.013	0.835 ± 0.01	0.287 ± 0.008
W3PP	0.384 ± 0.005	0.096 ± 0.003	0.930 ± 0.005	0.350 ± 0.004
W3PF	0.407 ± 0.006	0.133 ± 0.006	0.960 ± 0.005	0.397 ± 0.004
W3C	0.299 ± 0.009	0.041 ± 0.01	0.854 ± 0.01	0.224 ± 0.007
F WDS × PGPR	2.55 $^{n.s.}$	0.12 $^{n.s.}$	5.05**	7.88**

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

the effects of PP and PF strains were identical. In shoots, however, HYO content significantly increased with increasing WDS in all employed treatments. The results also showed that HYO content of shoots under W3 conditions for PF- and PP-inoculated plants were almost 12.4% and 8.9% higher than that of control plants, respectively. SCO content of shoots in all employed treatments had the same changes as roots, and mildly increased with increasing WDS only in PF-treated plants (Table 5). It seems that inoculation of *H. niger* plants with PF promoted HYO and SCO accumulation in both root and shoot organs. Shoot HYO yield decreased with increasing WDS in both *Pseudomonas*-treated and uninoculated control plants; however, the percentage reductions in PP- and PF-treated plants were lower than those in the uninoculated controls. Shoot SCO yield also decreased with increasing WDS in PP-treated and control plants, but was unchanged in PF-treated plants. The largest total alkaloid (HYO + SCO) yield was seen in PP-treated plants under W1 conditions mainly because of lower growth parameter reductions in comparison with the other treatments (Table 6).

4. Discussion

Rhizobacteria, including fluorescent pseudomonads, have substantial effects on plant growth, particularly under stress conditions, and play an important role in plant physiology

Table 6. Mean values for the alkaloids (HYO and SCO) yield in root and shoot (mean ± SD, n = 3) of *H. niger* inoculated with *Pseudomonas* strains at different water deficit stress levels.

Treatment	Root alkaloid yield (mg plant^{-1})		Shoot alkaloid yield (mg plant^{-1})		Total alkaloids
	HYO	SCO	HYO	SCO	yield (mg plant^{-1})
W1PP	4.172 ± 0.115	1.046 ± 0.056	14.572 ± 0.149	5.921 ± 0.095	25.711 ± 0.40
W1PF	3.790 ± 0.105	0.907 ± 0.042	13.591 ± 0.144	5.421 ± 0.088	23.709 ± 0.34
W1C	2.310 ± 0.145	0.492 ± 0.092	10.634 ± 0.192	3.524 ± 0.098	16.960 ± 0.43
W2PP	3.286 ± 0.097	0.953 ± 0.037	11.740 ± 0.067	4.872 ± 0.040	20.851 ± 0.19
W2PF	3.696 ± 0.111	1.094 ± 0.044	12.828 ± 0.041	5.192 ± 0.012	22.811 ± 0.09
W2C	1.487 ± 0.078	0.377 ± 0.057	7.660 ± 0.147	2.616 ± 0.056	12.140 ± 0.24
W3PP	2.690 ± 0.057	0.668 ± 0.032	9.244 ± 0.141	3.472 ± 0.082	16.074 ± 0.30
W3PF	3.301 ± 0.096	1.046 ± 0.044	11.351 ± 0.026	4.682 ± 0.008	20.398 ± 0.09
W3C	0.892 ± 0.056	0.123 ± 0.043	4.899 ± 0.090	1.281 ± 0.045	7.194 ± 0.17
F WDS × PGPR	11.09**	4.06*	13.74**	21.57**	24.34**

W1, W2, and W3: water deficit stress (WDS) 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.

by a combination of direct and indirect mechanisms (21). In our study, PGPR inoculation had positive effects on root and shoot growth indexes such as leaf number, leaf area, and leaf greenness both in the presence and absence of WDS. Moreover, root inoculation of *H. niger* with these PGPR decreased the severe negative effects of WDS on fine root growth. This effect could be attributed to the ability of PP and PF to release indole-3-acetic acid (IAA). Similarly, growth promotion of many plants with inoculation of PGPR has been reported previously (22,23). It has been suggested that direct mechanisms of PGPR involve secretion of plant growth regulators (auxins, cytokinins, and gibberellins), which enhance various stages of plant growth or synthesize enzymes that modulate plant growth and development (24). In the present study, the increase in root dry weight compared to shoot in both PP- and PF-treated plants under WDS treatments could be associated with multiple plant growth promoting traits such as phosphorus solubility, siderophore and hydrogen cyanide (HCN) production, auxin secretion, and ACC (1-aminocyclopropane-1-carboxylic acid) deaminase activity of PP and PF (Table 1), which change the assimilate allocation patterns in plants and affect the growth patterns in roots. As a result, *H. niger* root systems were larger and more branched, which was in agreement with findings reported by Potters et al. (25). Moreover, 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 (26). In indirect mechanisms, secondary bacterial metabolites are involved such as HCN and siderophore production that chelate iron and make it available to the plant root (21). It was recently reported that inoculation of wheat seedlings with *Mycobacterium phlei* significantly stimulated shoot growth (up to 32%), root growth (up to 6%), and dry matter contents (up to 52%) under saline conditions through its ability to produce different biologically active compounds, such as cell wall-degrading enzymes and the phytohormone auxin (27).

WDS, like many abiotic stresses, causes pronounced effects on RWC, chlorophyll, and proline content. WDS decreased RWC in the leaves of *H. niger* plants. The RWC values of pseudomonas-treated plants were higher than respective uninoculated control plants. This may be associated with the hydraulic nature of branch root junctions, which facilitate the radial flow of water, as previously reported by Kothari et al. (28). Yuwono et al. (29) reported that root proliferation enhancement and enhanced water uptake in inoculated drought-stressed rice plants may be induced by IAA. The advantageous effects of PGPR and common adaptation mechanisms of plants exposed to WDS are always mutually related to exceptional changes in root morphology such as root dry weight and root branching (30). Marcelo et al. (22) demonstrated that common bean (*Phaseolus vulgaris* L.) treated with *Azospirillum brasilense* Cd (N_2-fixing and PGPR) had enhanced root length and area compared to uninoculated controls, resulting in root systems with longer and thinner roots. Plants with very fine roots are more effective for water and mineral uptake due to higher root specific surface area. Our results indicated that the PF strain had higher efficiency than the PP strain in plants growing under moderate (W2) and severe (W3) WDS conditions. Strain PF (with a strong phosphorous solubilizing activity) was isolated from rainfed (dry land farming) wheat rhizosphere, where water is restricted and dry periods often take place. This may explain the superiority of PF under a limited water supply compared to PP (which has no phosphorous solubilizing activity), which was isolated from irrigated wheat rhizosphere (Table 1). Such results were also in accordance with a previous report by Mayaka (31). Although ACC deaminase activity was not observed in PF characteristics (Table 1), this strain improved root and shoot growth compared to PP and uninoculated control plants under severe WDS. Several studies have supported the beneficial effects of ACC deaminase activity of PGPR on plants growing under stress conditions (21). This may be attributed to other bacterial abilities. It is obvious that ACC deaminase activity is not the only factor responsible for root growth enhancement. ACC deaminase activity might have produced better root growth in the initial stages of crop growth in stress conditions (32), whereas in the present study water stress started at 45 DAS. In our study, WDS reduced the contents of chlorophyll *a* and *b*. A reduction in chlorophyll content has also been reported in certain plant species exposed to drought stress, such as periwinkle (33). Inoculation of *H. niger* plants with PGPR partially eliminated the deleterious water stress effects on growth and chlorophyll content, as was evident from the data documented in Tables 2 and 3. Application of PGPR on pea (*Pisum sativum* L.) was found to be very effective in decreasing the drought stress effects on the chlorophyll content (34). In research by Karakurt et al. (35) the effects of 4 plant growth promoting rhizobacteria, alone and in combination, on growth and chemical characteristics of sour cherry was studied, and it was reported that all the tested bacterial strains had a great potential to increase plant vegetative growth and indirectly affect chemical characteristics. Our results in this case are in accordance with findings reported by Jaleel et al. (23) in *Catharantus roseus*. Our findings suggested that using native PGPR strains to induce and enhance pigment biosynthesis could be a biological strategy for improving drought tolerance in plants growing under water stress conditions. Rhizosphere and plant dependent mechanisms have been proposed for the stress tolerance mediated by PGPR (36).

Studies have shown that WDS is a major environmental factor limiting the productivity of plants (biomass accumulation) and may cause damage to plant tissues, especially roots, through the formation of ROS such as H_2O_2, O^{-2} and OH^-, due to high susceptibility of root meristem activity to ROS (9). In order to decrease the deleterious effects of ROS, antioxidant promoting systems are required, such as PGPR application. Plants have developed physiological and biochemical mechanisms to respond and adapt to this stress in order to survive. The carbon/nutrient balance (CNB) and growth differentiation balance (GDB) hypotheses predict a trade-off between growth and defense systems. Because WDS reduces plant growth, the carbon fixed during photosynthesis can be used for the production of secondary metabolites (37). Although WDS is the major limiting factor on crop plant growth and biomass production, enhancement of secondary plant products, solute accumulation, and enzyme activities are considered positive effects of limited water supply in medicinal plants (38).

Proline accumulation is a very common response in plants exposed to WDS; it contributes to osmotic adjustment and protects the structure of macromolecules and cell membranes during WDS (39). Meloni et al. (40) suggest that amino acids such as proline acts as an organic nitrogen reserve in plant metabolism, as a readily available source of energy, and as possible precursors of alkaloid formation. This could explain why the plants inoculated with the PF strain produced higher contents of tropane alkaloids.

WDS is known to increase O^{-2} production in chloroplasts. SOD is an enzyme that catalyzes the conversion of the O^{-2} to O_2 and H_2O_2 (41). Enhanced SOD activity of roots and leaves under severe WDS conditions for PGPR-treated plants may be interpreted as a direct response to augmented O^{-2} generation, particularly in chloroplast compartments. In our study, SOD activity of roots and leaves showed a significant increase with increasing WDS and PGPR application. The important point here is a decrease in SOD activity in both roots and leaves of control plants under severe WDS, which may reflect the low ROS scavenging capacity and increased damage to plant parts under this condition.

POX showed an increase in activity in response to WDS in root and leaf parts of both PGPR-treated and control plants. This enzyme is involved in the scavenging of H_2O_2, growth, and developmental processes (42). Thus, our results show that drought-related oxidative stress upregulated the activity of POX in *H. niger* root and leaf organs. This might be an important protection mechanism in *H. niger* plants against the excessive increase of H_2O_2 during WDS. These results are also consistent with those reported by Kohler et al. (43).

CAT, which is localized in peroxisomes, dismutates H_2O_2 into H_2O and O_2 (41). According to our results, although the activities of SOD and POX of roots and leaves in PGPR-treated plants were upregulated by WDS, CAT activity decreased in all treatments, except for in PF-inoculated plants (Table 4). The decline in CAT activity is regarded as a common response to many stresses. The reduction of CAT activity is supposedly due to the inhibition of enzyme synthesis or change in the assembly of enzyme subunits under stress conditions. It may also be associated with degradation caused by induced peroxisomal proteases or may be due to the photo-inactivation of the enzyme (44). Our results are in agreement with the findings reported by Kohler et al. (43) in lettuce plants, which showed that the greater activity of antioxidant CAT and accumulation of under severe drought conditions were recorded when inoculated with PGPR, *Pseudomonas mendocina,* and arbuscular mycorrhizal fungi.

It is suggested that the overexpression of SOD, if accompanied by enhanced H_2O_2 scavenging mechanisms like CAT and POX activities, has been considered an important antidrought mechanism to cope with oxidative stress during WDS conditions (43). Generally, SOD and POX in roots and leaves showed simultaneous induction and decline, which may be due to their co-regulation, which agrees with the finding reported by Shigeoka et al. (45). Plants inoculated with PF and PP strains were more hydrated than the control untreated plants. These results demonstrated that the PGPR treatment influenced the extent of WDS and that PF efficiently protected the host plants against the detrimental effects of WDS. Likewise, our results indicated a significant role of PGPR, particularly in the application of PF, providing a protective mechanism by scavenging ROS and increasing the activities of SOD and CAT against drought-dependent oxidative damage.

The use of elicitors is an effective means employed to increase the production of important alkaloids. PGPR and osmotic stresses are classified as biotic and abiotic secondary metabolites elicitors in medicinal plants (46). In our study, rhizobacteria have ability to produce growth regulators such as IAA (Table 1), which could act as elicitors on tropane alkaloid biosynthesis under WDS, resulting in raising alkaloid yield and content. According to our results, PP-treated plants under W1 conditions had a higher proportion of fine roots compared to other treatments, resulting in high HYO and SCO yield in the roots and shoots. Nakanishi et al. (47) found that root secondary growth is a significant factor determining tropane alkaloid composition, which varied based on root diameter. The plant growth promoting properties of PGPR could be the driving force for the alkaloid yield increment in this study. WDS had also positive effects (but less than PGPR) on alkaloid content of plant roots and shoots. Increases in free amino acids, soluble proteins, and soluble

carbohydrates are among the factors contributing to higher alkaloid contents in plants experiencing WDS (48). However, it significantly reduced plant biomass, which is a key determinant of alkaloid yield per plant. In the current study, shoots showed higher alkaloid accumulation than roots. It is well established that the root is the location of tropane alkaloid biosynthesis in Solanaceae family plants, but they may be transported to the aboveground parts of the plant (49).

The results of this study suggest that *H. niger* plants with an inoculation of PF under moderate WDS (W2), in addition to having appropriate amounts of each alkaloid content and yield, also have the largest value of SCO, which is the indicator of tropane alkaloid quality. Thus, inoculation of *H. niger* roots with PF strain not only alleviated the deleterious stress effects on plant growth to some extent, it also improved the growth parameters under WDS and increased elicitation of tropane alkaloid production.

Our results suggest that inoculation of *H. niger* plants with PP and PF strains stimulated the activities of antioxidant enzymes, increased proline accumulation, and remarkably improved the alkaloid content and yield of root and shoot organs. WDS improved the content of these alkaloids, but retarded the growth parameters, resulting in low alkaloid yield. Moreover, plants treated with the PP strain had the highest alkaloid yield under low WDS conditions, whereas under moderate and severe WDS, PF was the effective strain. Therefore, application of suitable PGPR strains should be based on multiple plant growth promoting characteristics and their ecological adaptation with respect to the abiotic stress of the host plant. Integrative use of effective PGPR strains (biotic elicitors) and abiotic elicitors such as WDS appears to be an encouraging new method and ecofriendly strategy for increasing tropane alkaloid yield and quality in *H. niger* plants. In the future, we will conduct studies on correlations between the tropane alkaloid biosynthesis and the activities of putrescine N-methyltransferase and hyoscyamine 6β-hydroxylase, enzymes that are involved in the biosynthesis of tropane alkaloids, with inoculation of PGPR under WDS conditions.

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