ALLEVIATION OF BLEACHING HERBICIDE TOXICITY BY PGPR STRAIN ISOLATED FROM WHEAT RHIZOSPHERE

Maissa BOURAHLA*, Réda DJEBBAR*, Yahia KACI*, Ouzna ABROUS-BELBACHIR*

*University of Science and Technology Houari Boumedienne (USTHB), Algiers, Algeria, Faculty of Biological Sciences, Laboratory of Biology and Physiology of Organisms
Corresponding author: Maissa Bourahla, University of Science and Technology Houari Boumediene, BP 32, El Alia 16111 Bab Ezzouar, Algiers-Algeria, phone: +213 555581024, e-mail: mbourahla74@gmail.com

Abstract. Plant Growth Promoting Rhizobacteria (PGPR) can play important role in agriculture. The objective of this study was to assess the ability of the isolated strain *Pseudomonas putida* to improve physiological and biochemical parameters of durum wheat seedlings (*Triticum durum* Desf. Var. Mohamed Ben Bachir) and helped them to withstand bleaching herbicide the norflurazon. At 10^{-4} M, the norflurazon induced the bleaching of seedlings (photobleaching). At the physiological level, it caused the inhibition of carotenoids (0.03±0.01 mg g^{-1}), chlorophylls (0.15±0.03 mg g^{-1}) and carbohydrates (0.28±0.07 mg g^{-1}) contents. At the cellular level, carotenoids defiency angendred by the norflurazon induced oxidative stress which manifested by an increase of MDA content (32.33±2.08 µmoles g^{-1}), a lipid peroxidation marker, as well as a sharp increase in the electrolytes leakage (63.7±6.01 %) sign of membrane systems deterioration. The norflurazon also decreased the catalase (7.13 µmol H_2O_2 $mn^{-1}mg^{-1}$of proteins) and glutathion S-transferase (2 nanomol $min^{-1}mg^{-1}$ of proteins) activities in the leaves of durum wheat seedlings. The strain of *Pseudomonas putida* with inherent phosphate solubilizing activity, auxins (IAA) synthesis and EPS production was inoculated to the wheat seedlings in the presence and absence of norflurazon. It had eased phytotoxic effects of norflurazon on wheat seedlings and improving theirs physiology and their antioxidant mechanisms. The bacteria strain used would so represent a valuable partner in the future agriculture by its bioprotective and biostimulating properties.

Keywords: *Pseudomonas putida*; PGPR (Plant Growth Promoting Rhizobacteria); norflurazon; oxidative stress; *Triticum durum.*

INTRODUCTION

Herbicides are chemicals that are used to kill plants adventitious. These latter penalize crops yield by a competition on the space occupation, water exploitation, light and use of ground nutritive elements. The use of herbicides in agriculture is not without problems. Some herbicides contain chemical compounds that are extremely toxic and persist in the environment. The persistent nature of some chemicals makes them to accumulate in the environment affecting the beneficial microorganisms and their important physiological activities to soil fertility and ultimately influence the plant growth [36, 37].

Norflurazon [SAN 9789, 4-chloro-5-(methylamino)-2-(3-trifluoromethylphenyl)-pyridazin-3(2H) one] is a selective pre-emergent herbicide widely used to control germinating annual grasses and broadleaf weeds in fruits, vegetables, nuts, cotton, peanuts and soybeans [2]. It's considered the most effective herbicide of pyridazine derivatives. Norflurazon is a potent inhibitor of phytoene desaturase a key enzyme of biosynthetic pathway of carotenoids [7] and of ω-9 fatty acid desaturase [1]. This herbicide generates oxidative stress by increasing the production of Reactive Oxygen Species (ROS) that can damage cellular components [20, 23]. The ROS such as H_2O_2, O_2^- and OH may accumulate during herbicide stress and cause damaging effect in chloroplasts including chlorophyll destruction, lipid peroxidation and protein oxidation [23]. A balance between oxidant and antioxidant intracellular systems is hence vital for cell function, regulation, and adaptation to diverse growth conditions.

The rhizosphere is a zone of intense microbial and biochemical activity due to organic and mineral nutrients plants exudation. Bacteria that colonize the rhizosphere and affect the plant growth and yield of commercially important crops are denominated as Plant Growth Promoting Rhizobacteria (PGPR) [46]. These beneficial bacteria have been recognized in the root health maintenance, nutrient uptake and environmental stresses tolerance to enhance plant growth. They promote the growth indirectly by the reduction or prevention of the action of plant pathogens, or directly via phosphorus solubilization, nitrogen fixation, iron sequestration by siderophores, phytohormone production (auxin, cytokinin, or gibberellin), and/or enzymatic lowering of plant ethylene levels [18].

Recent studies have focused on the use of plant-PGPR interaction to reduce the intensity of the stress due to the application of chemical fertilizers and pesticides, which often pollute the environment [41]. *Pseudomonas*, especially fluorescent P*seudomonas* group has emerged as the largest potentially most promising group of PGPR [32]. Previous studies [26, 29 ,34] led to the isolation and characterization of the species *Pseudomonas putida.* In this study we evaluated the capacity of *P. putida* strain isolated from wheat durum rhizosphere to alleviate chemical stress induced by the norflurazon in durum wheat seedlings (*Triticum durum*).

MATERIAL AND METHODS

Isolation of Fluorescent *Pseudomonas* Strain

Rhizosphere soil sample of wheat durum cultivated in Oued Smar experimental station (Algiers, noth of Algeria) was collected. 10 g of rhizosphere soil was transferred to a 250 mL Erlenmeyer flask containing 90

mL sterile distilled water and shaken (120 rpm for 30 min). 0.1 mL of each diluted suspension (10^{-4} to 10^{-7}) was spread plated on a selective medium of fluorescent *Pseudomonas* King B (20 g peptone, 10 mL glycerol, 1.5 g K_2HPO_4, 5 g $MgSO_4$, 5 g agar, 1000 mL H_2O, pH 7-7.2) [24]. Plates were incubated at 28-30° C during 24-72 hours. Fluorescence of *Pseudomonas* colonies was observed on King's B medium under UV exposure. The isolate was tested for morphology, motility and Gram strain by phase contrast microscopy. Catalase and oxydase activities were performed. API 20NE strip (Bio Mérieux, France) was used for a preliminary biochemical characterization of the fluorescent *Pseudomonas* strain and the species was identified using the API database.

Promoting Growth Activities

Mineral phosphate solubilization

The quantitatif test was conducted *in vitro* by inoculating the bacteria in the NBRIP agar medium [39] amended with precipitated tricalcium phosphate (glucose 10 g, $(NH_4)_2SO_4$ 0.1 g, Ca_3 (PO_4) 2.5 g, $MgSO_4 \cdot H_2O$ 0.25 g, $MgCl_2 \cdot 6H_2O$ 5 g, KCl 0.2 g, H_2O 1000 mL). Bacterial culture corresponding to 10^8 cfu mL^{-1} was spot-inoculated on the surface of NBRIP Agar medium. Plates were incubated at 28-30 °C for 7 days. The total diameter (colony + halo) and the diameter of the colony were then determined at the end of incubation period (15 days). The Solubilisation Index
(SI) = halo diameter (mm) / colony diameter (mm) was calculated. Strain was classified based on it's SI as demonstrating low (SI < 2.00), intermediate (2.00 < SI < 4.00) and high (SI > 4.0) solubilization capacities [30].

Indole-3-acetic acid (IAA) production

Production of IAA by isolated strain was estimated according to colorimetric technique proposed by Gordon and Weber [16]. 1 mL of bacterial culture was inoculated in 100 mL of Tryptic Soy Broth (TSB) without and with tryptophan concentrations (0.05%, 0.1% and 0.5%) and shaking in the dark for 48 h at 160 rpm and 30 °C. After centrifugation, the determination of bacterial auxin (IAA) levels in supernatant is based on the reaction of Salkowski and the absorbance was measured at 530 nm. The quantity of IAA (µg mL^{-1} of TSB medium) was determined by using a standard graph of IAA.

Exopolysaccharide synthesis

Isolated strain was screened for exopolysaccharide (EPS) production by growing it on Yeast Extract Mannitol Agar medium or YEMA [51] (Mannitol 10 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaCl 0.1 g, Yeast extract 0.5 g, Agar 15 g, 1000 mL H_2O) changing the carbon substrate mannitol in the first case by the sucrose and in the second case by the glucose. Plates were incubated at 28-30 °C for 7 days.

Experimental Protocol

Material used

The rhizobacteria was grown in Tryptic Soy Broth on a rotary shaker at 160 rpm for 24 h. After centrifugation, the cell pellet was used as inocula [OD_{600}: 0.5] for plant assays. *Triticum durum* seeds of the same weight class (modal class) were surface-sterilized in 5% calcium hypochlorite solution containing 1 mL of ethanol for 20 min and thoroughly rinsed with sterile distilled water. For germination, seeds were deposited on TSA medium surface and placed in an incubator at 25 °C. Norflurazon sterile solution 10^{-4}M was prepared by the method of dilution in sterile distilled water.

Plant culture

Three germinated seeds of durum wheat were deposited in each test tube containing glass beads as solid support and 12 mL of KNOP sterile mineral medium [$Ca(NO_3)_2$ 1 g, KNO_3 0.25 g, $MgSO_4$ 0.25 g, KH_2PO_4 0.25 g, $FeCl_3$ 0.001 g, 1000 mL H_2O, pH 5-5.8]. This experience includes four batches, carried out in three replicates: control batch (C), control inoculated with bacteria (CP), control treated with Norflurazon 10^{-4} M (N), control inoculated and treated with the same herbicide concentration (NP). The test tubes were then placed in the growth chamber at 25 °C, 16 h-photoperiod.

Plant Analyzes

Photosynthetic pigments content

Chlorophyll (a+b) and carotenoids contents were determined according to the method described by Lichtenthaler [27]. Concentration of chlorophyll (Chl) and carotenoids expressed in mg g^{-1} of fresh vegetable matter were calculated after the absorbances were measured at λa = 663 nm (chlorophyll a), λb = 647 nm (chlorophyll b) and λc = 470 nm (carotenoids).

Ethanolosoluble sugars content

The ethanolosoluble sugar was determined by the anthrone method developed by Mc Ready et *al.* [33]. The ethanolosoluble sugar contents were determined based on calibration standard prepared using glucose (100 µg mL^{-1}).
Results were expressed as mg of glucose equivalents for g of fresh weight of wheat durum.

Estimation of lipid peroxidation

The method of assessing the malondialdehyde (MDA) in plant tissue was presented by Heath and Packer [19]. The MDA concentration was calculated

using its extinction coefficient equal to 155 $mM^{-1}cm^{-1}$. Results were expressed in
µmol g^{-1} of fresh vegetable matter.

Estimation of cell membrane integrity by electrolyte leakage measurement

Evaluation of electrolyte relative leakage (expressed in %) in leaf pieces was determined using the method of Bajji et *al.* [4]. It was calculated according to the formula:

Electrolyte Relative Leakage (%) = (EC / ET) x 100

where EC and ET refer to the initial and final conductivity measurement, respectively.

Catalase activity assay

Catalase activity was measured by the method of Dorey et *al.* [12]. The assay was based on the amount of H_2O_2 decomposed, determined by measuring absorbance change at 240 nm. The enzyme activity can be calculated using extinction coefficient of H_2O_2 (ε = 36 Mm cm^{-1}). Protein concentrations were estimated according to Bradford's method [6] using Bovine Serum Albumin (BSA: 2 mg mL^{-1}) as a standard. Catalase activity was expressed in µmol of H_2O_2 min^{-1} mg^{-1} of protein.

Glutathione S-Transferase (GST) activity assay

The measurement of glutathione S-transferases (GST) activity consists in supplying at the enzyme the standard model substrate (1-chloro-2.4-dinitrobenzene; CDNB, which reacts easily with numerous forms of GST) and glutathione. The reaction was measured by observing the conjugation of CDNB substrate with reduced glutathione (GSH). GST activity was determined spectrophotometrically at 340 nm as described by Habig et *al.* [17]. Specific activity of GST was expressed as µmol of formed product (GS-DNB) min^{-1} mg^{-1} of protein.

Statistical Analysis

Analysis of Variance (ANOVA) was used to determine the significance of treatment effects for all tests. Means comparisons were made by using the Excel 2007 software to the thresholds of significance: not significant (P> 0.05); *: significant at 0.05; **: very significant at 0.01; ***: highly significant at 0.001.

Note that black asterisks indicate the difference between the control batch (C) and the other ones and red asterisks indicate the difference between non inoculated (C, N) and inoculated (CP, NP) batches respectively in each experimental condition.

RESULTS

Isolation and Identification of Fluorescent *Pseudomonas* Strain

Pigment fluorescent production is a characteristic frequently found in fluorescent *Pseudomonas* species (Fig. 1). On the basis of the morphological, physiological tests and biochemical characterization (Tab. 1), determined by means of API 20NE strips, the strain was presumptively identified as *Pseudomonas putida.*

Pseudomonas putida staining is negative. The figure 2 illustrated a pink short-rods (bacilli).

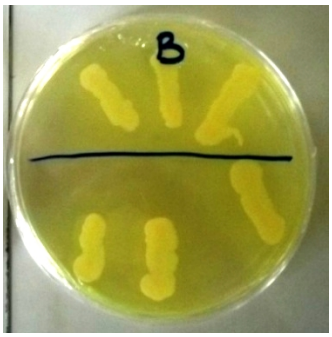

Figure 1. Aspect of fluorescent isolated colonies on King B medium

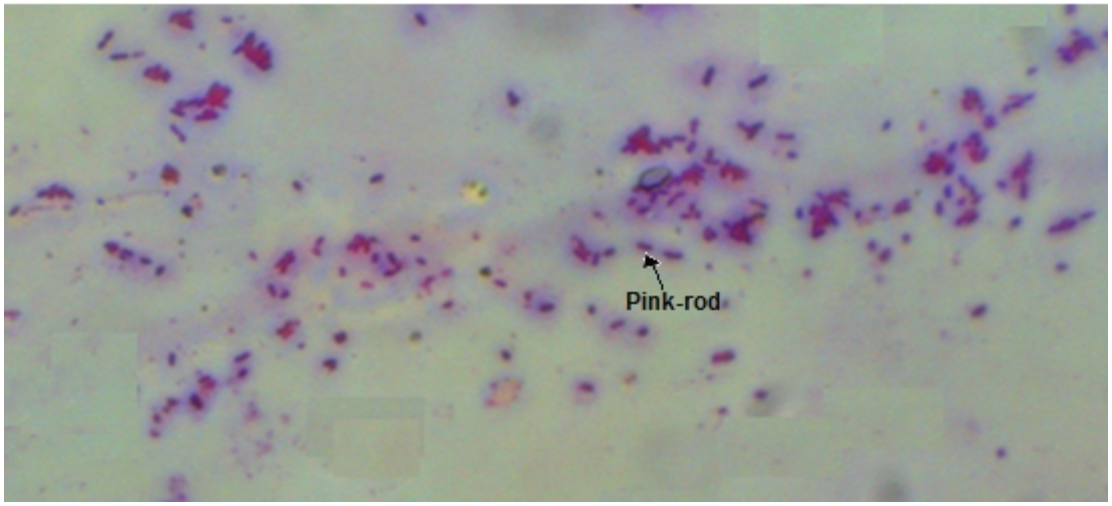

Figure 2. Gram staining of *P. putida* strain

Table 1. Morphological and biochemical characteristics of *Pseudomonas putida* strain

Shape	Motility	Gram reaction	Catalase test	Oxidative test			Fluorescent pigment
Rod	Motile	-	+	+			+
API 20 NE Tests							
Denitrification	Indol production	Glucose fermentation	Arginine	Urease	Esculin	Gelatine	p-Nitro-phenyl-β-D-galacto- pyranoside
-	-	-	+	-	-	-	+
Glucose	Arabinose	Mannose	Mannitol	N-acetyl-D-glucosamine			Maltose
+	-	-	-	-			-
Gluconate	Caprate	Adipate	Malate	Citrate			Phenylacetate
+	+	-	+	+			+

(+): Positive test; (–): Negative test

Promoting Growth Activities

Mineral phosphates solubilization

The production of clear zones around the colonies (Fig. 3) indicated that the isolated strain was able to dissolve the tricalcium phosphates present in the NBRIP medium of Nautiyal [39]. At the end of the experimental evaluation, SI was 3.

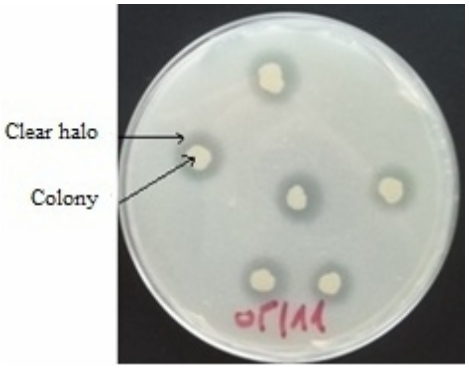

Figure 3. Bacterial isolate showed medium zone of solubilization on NBRIP agar medium

Production of indole 3-acetic acid (IAA)

The isolated strain was tested for the quantitative estimation of IAA in the presence of different concentrations of tryptophan (0 % to 0.5 %). With no addition of tryptophan, production of IAA was not observed. The maximum IAA production of 495 µg mL^{-1} of Tryptic Soy Broth was obtained when 0.5% of L-tryptophan was used (Fig. 4).

Production of exopolysaccharides

Macroscopic observation of the bacterial colonies on three culture media used for the EPS production showed that they appeared mucous in the presence of sucrose (YESA medium) (Fig. 5).

Morphology and Growth

Durum wheat seedlings (*Triticum durum*) treated with norflurazon at 10^{-4} M showed modifications of the morphological appearance manifested by bleaching of the leaves: foliar parts loosed their green color to become partially or completely white (photobleaching phenomenon) and reduces size of the treated plants (Figs. 6A, B).

Physiological and Biochemical Parameters

Chlorophylls (a+b)

The analysis of variance (ANOVA) indicated that there was a significant difference in bacteria factor and herbicide factor. Norflurazon at 10^{-4} M led in a highly significant decrease (-86.11%) of the total chlorophyll content (a + b) in comparaison with the control seedlings (C) (Fig. 7A). We noticed a significant increase (+293.33%) of the total chlorophyll content (a+b) in inoculated and norflurazon-treated seedlings (NP) compared to norflurazon-treated seedlings (N).

Carotenoids

Results Analysis by ANOVA test revealed significant effect of bacteria factor and herbicide factor. A highly significant fall of carotenoids content (-86.36%) was recorded in norflurazon-treated seedlings (N) (Fig. 7B). *Pseudomonas putida* caused a very significant increase (+266.66 %) of carotenoids content in seedlings (NP) relative to that of the norflurazon treated seedlings (N).

Ethanolosoluble sugar content

The ANOVA performed on ethanolosoluble sugars revealed statistically significant effects of the bacteria factor and norflurazon factor. Our results indicated that the quantity of ethanolosoluble sugars reduced very significantly in seedlings norflurazon-treated (-44%). The rhizobacteria strain increased in highly significant way ethanolosoluble sugars amount in plants control (Fig. 8).

Lipid peroxidation

Analysis of variance revealed a significant difference in the bacteria factor, herbicide factor and the interaction factor (bacteria× herbicide). The results showed that treatment of norflurazon (10^{-4} M) increased in a highly significant way the contents of

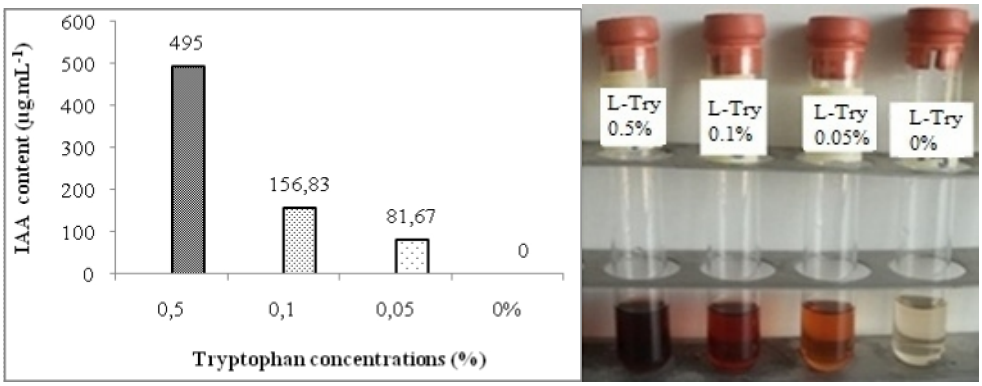

Figure 4. Production of auxin (IAA) by the isolated strain in presence of different concentrations of tryptophan

Figure 5. Production of exopolysaccharides by isolated strain on Yeast Extract Mannitol Agar (A) Yeast Extact Glucose Agar (B) and Yeast Extract Sucrose Agar (C) mediums

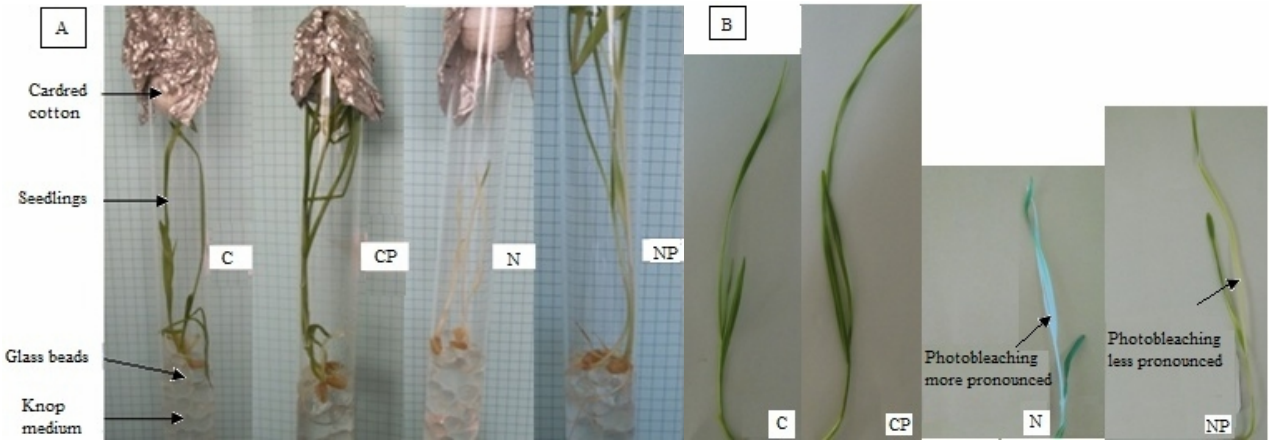

Figure 6. Wheat seedlings morphology (A) and impact of *Pseudomonas putida* on aerial part morphology of durum wheat seedlings not treated or trated with norflurazon (10^{-4} M) (B). C = controls; CP = Inoculated seedlings; N = seedlings + norflurazon 10^{-4} M, NP = Inoculated seedlings + norflurazon 10^{-4} M

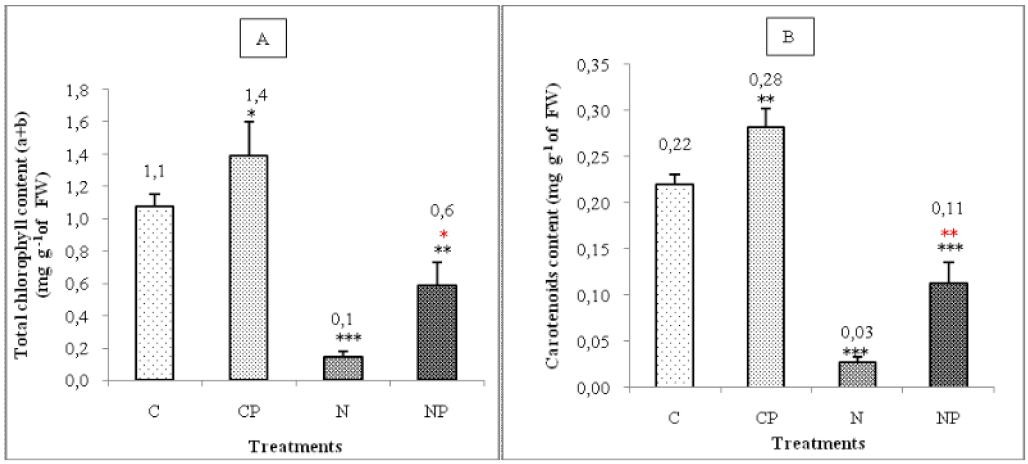

Figure 7. Impact of *Pseudomonas putida* on total chlorophyll content (A) and carotenoids content (B) of wheat durum seedlings not treated or treated with norflurazon (10^{-4} M). Not significant (P> 0.05); *: significant at 0.05; **: very significant at 0.01; ***: highly significant at 0.001

MDA (N) (+173.28%) (Fig. 9A). In contrast, the bacterial strain *Pseudomonas putida* caused a significant decrease in MDA content in plants (CP) (-27.47%) compared with plants control while it caused a very significant decrease in plants (NP) (-29.87%) compared with plants (N).

Cell membrane integrity

The results showed significant differences of the bacteria factor and herbicide factor. Norflurazon at 10^{-4} M increased very significantly the relative electrolyte leakage in the plants contol (N) (+82.47%) (Fig. 9B). In unstressed seedlings, the rhizobacteria strain generated significant decline (-32.45%) of the relative electrolyte leakage compared to that of plants control (C). The electrolytes leakage decreased very significantly by PGPR strain in plants (NP), indeed reduction rate of (-27.39%) compared with seedlings norflurazon-treated (N) was recorded at the concentration 10^{-4} M.

Catalase activity

The ANOVA test showed a significant effect of the bacteria factor, herbicide factor and that of their interaction factor (bacteria × herbicide). The measurement of catalase activity in seedlings treated with norflurazon (N) showed a significant decrease (-22.07%) of the latter compared to that of control plants (C).

In unstressed seedlings, the activity of catalase increased in highly significant way (+156.5%) under the influence of the PGPR strain. In seedlings (NP), catalase activity presented a highly significant enhancement (+176.85%) compared to seedlings (N) (Fig. 10).

Glutathione S-Transferase (GST) activity

The ANOVA revealed a significant effect of herbicide factor and no significant effect of rhizobacteria and the interaction factors. In treated plants (N) the GST activity decreased in very significant way (-50%) as compared to the plants control (C). The rhizobacteria strain enhanced GST activity in control seedlings by highly significant manner (+100%). In wheat seedlings inoculated with bacteria strain under the norflurazon, the GST activity increased significantly by (+50%) as compared to the uninoculated seedlings treated by the same concentration (Fig. 11).

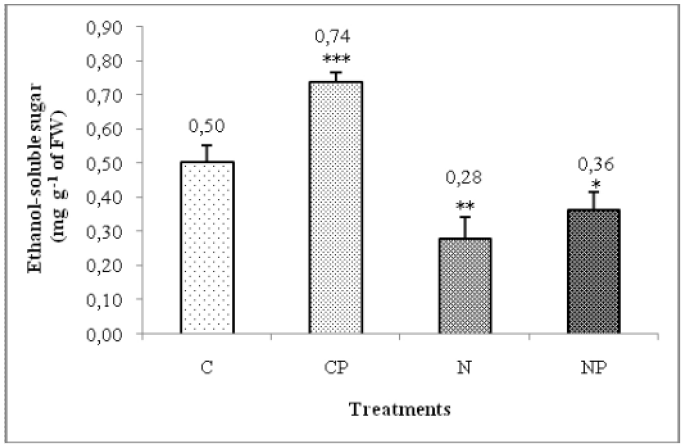

Figure 8. Impact of *Pseudomonas putida* strain on ethanolosoluble sugar in wheat durum seedlings not treated or treated with norflurazon (10^{-4} M). Not significant (P> 0.05); *: significant at 0.05; **: very significant at 0.01; ***: highly significant at 0.001

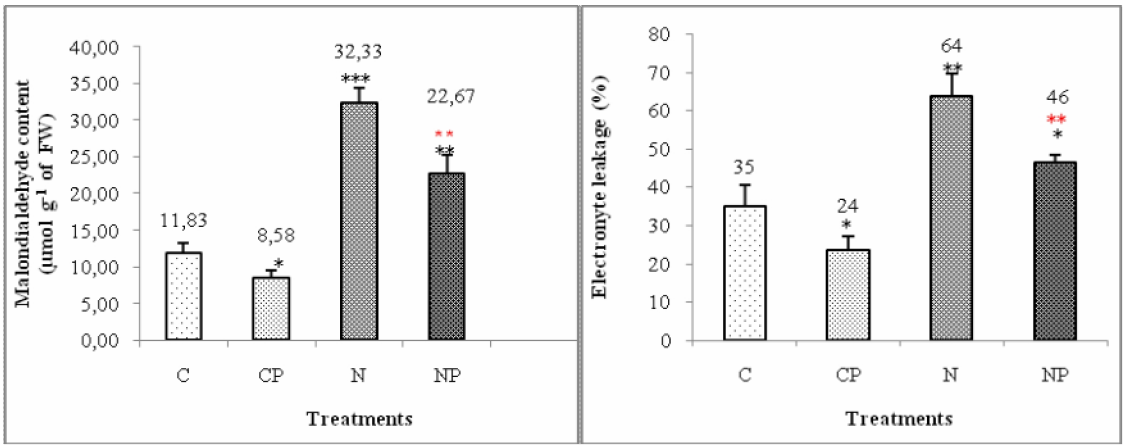

Figure 9. Impact of *Pseudomonas putida* strain on thiobarbituric acid-reactive substances those MDA content (A) and the percentage of relative electrolyte leakage (B) in wheat durum seedlings not treated or treated with norflurazon (10^{-4} M). Not significant (P> 0.05); *: significant at 0.05; **: very significant at 0.01; ***: highly significant at 0.001

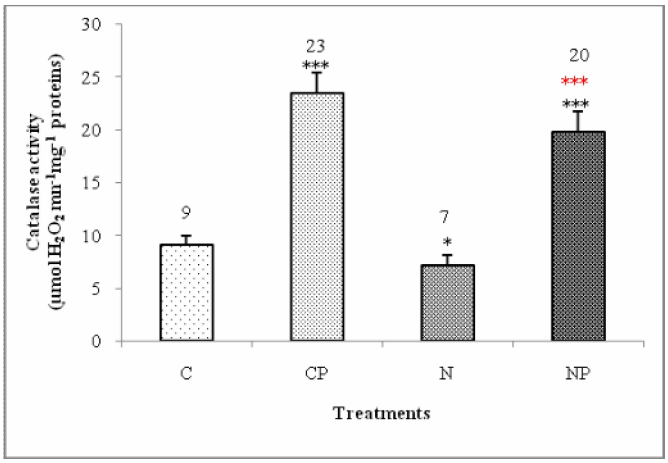

Figure 10. Impact of *Pseudomonas putida* strain on catalase activity in wheat durum seedlings not treated or treated with norflurazon (10^{-4} M). Not significant (P> 0.05); *: significant at 0.05; **: very significant at 0.01; ***: highly significant at 0.001

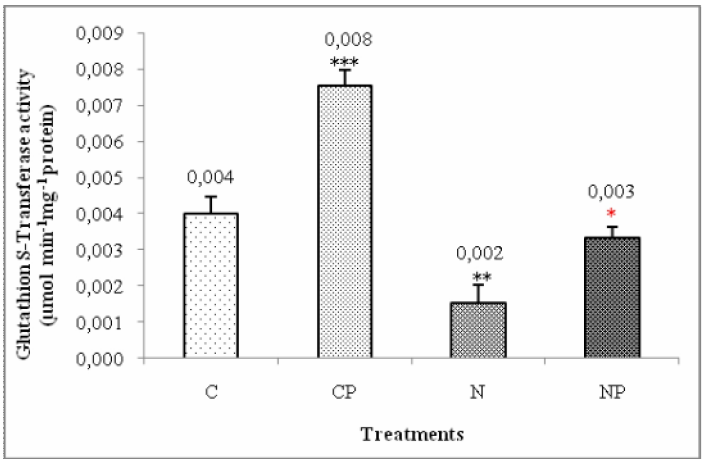

Figure 11. Impact of *Pseudomonas putida* strain on Glutathione S-Transferase activity in wheat durum seedlings not treated or treated with norflurazon (10^{-4} M). Not significant (P> 0.05); *: significant at 0.05; **: very significant at 0.01; ***: highly significant at 0.001

DISCUSSION

Large researches on wide variety of rhizosphere soil showed that rhizobacteria belonging to fluorescent *Pseudomonas* group were capable of colonizing the wide range of plants rhizosphere [32, 47]. Studies have enabled isolation and characterization of *P. putida* species. Thus, according to Lemanceau et *al.* [26], *P. putida* was the dominant species in the rhizosphere of corn; it was the most abundant of the *Pseudomonas* group in the rhizosphere flax, potato [29] and rapeseed *Brassica napus* [34]. The strain of *Pseudomonas putida* gave a yellow - green fluorescence in the King B medium. Indeed, fluorescents *Pseudomonas* are characterized by the ability to synthesize siderophores called pseudobactines or pyoverdins in iron deficiency situation.

Solubilization of the tricalcium phosphate by the isolated strain on the NBRIP medium could be related to the ability of these bacteria to release metabolites such as organic acids [15]. Lifshitz et *al.* [28] demonstrated that rape *Brassica campestris L.* inoculated with *P. putida* GR12-2 strain had phosphorus content greater than the uninoculated control. The production of exopolysaccharides has been shown in the YESA medium. Fett et *al.* [14] showed that members of fluorescents *Pseudomonas* group associated with plants such as *P. fluorescens*, *P. aeruginosa* could produced a wide range of EPS.

Indole acetic acid (IAA) is one of the most physiologically active auxins [3]. IAA is a metabolite derived from tryptophan (Trp) by many Trp-dependant and Trp-independent pathways in plants and bacteria [42]. Our bacterial culture released greater quantities of IAA in the presence of a physiological precursor, tryptophan, in a culture medium. It is known that IAA production of PGPR strains ranges in response to tryptophan addition from 5-10 to 100-200 mg IAAmL^{-1} and higher, depending on exogenous tryptophan concentration [49]. Leinho and Vacek [25] reported IAA production by *Pseudomonas* and *Acinetobacter* isolated from wheat and rye rhizospheres ranging from 0.01 to 3.98 mg mL^{-1}.

Norflurazon is an inhibitor of the biosynthetic pathway of carotenoids. The loss of carotenoids resulted in photo-oxidation of chlorophylls following the installation of oxidative stress [52]. Our results were in agreement with those of many authors. Jung [21] observed a decrease in the content of chlorophylls and carotenoids in seedlings of *Arabidopsis thaliana*

treated with norflurazon. The reduction of photosynthetic pigments content by the norflurazon affected carbohydrate metabolism. The decrease in ethanol-soluble sugars is due to the photosynthesis inhibition following the suppression of the expression of certain nuclear genes encoding plastid proteins such small sub-unit Rubisco (Ribulose -1,5-diphosphatecarboxylase / oxygenase) or the Cab gene encoding the complex chlorophylls a-b / proteins; expression of these genes is suppressed in depigmented seedlings after a signal interruption from the chloroplast toward the core. These results were in line with those reported by Sagar et *al*. [43] working on peas *Pisum sativum* after treatment with norflurazon.

The norflurazon caused deleterious effects at the cellular level by alteration of the membranes via lipid peroxidation; this alteration was assessed by the production of the malondialdehyde (MDA) a reactive substances to thiobarbituric acid (TBARS) and electrolytes leakage. Indeed, the energy of the excited chlorophyll is captured by the molecular oxygen which promotes the production of reactive oxygen species. These entail alteration of cell membranes which are rich in polyunsaturated fatty acids [1]. MDA is a highly toxic aldehyde; it is known to be a potential biomarker of oxidative stress and an increase in its concentration indicated an induction of oxidative stress by norflurazon [13]. The results that we obtained showed an increase in malondialdehyde (MDA) content and the relative ion leakage under norflurazon at 10^{-4} M. We also observed a positive correlation between the increase of the MDA content and the relative leaking of electrolytes. The loss of integrity of the cellular membrane of durum wheat seedlings, suggested that the peroxidation of lipids extended from components of the cell, as chloroplasts, in the plasmic membrane.

In presence of herbicides, the plants develop different detoxification systems to minimize and prevent the appearance of often irreversible damages to the cell [45]. Catalase (CAT) is an antioxidant that has the sole function of neutralizing H_2O_2 to form water and oxygen. It is located mostly in peroxisomes and glyoxisomes and plays a key role in protection against oxidative stress. Our results showed a significant decrease in catalase activity in the presence of high concentration of the norflurazon. This reduction let suggest that this enzyme was inactive in the case of a stress induced by severe photoinhibition [35]. *Tsun-Thai* et al. [50] reported that the decrease of the catalase activity of Berangan treated by the paraquat (family of pyridines) inducing oxidative stress in the chloroplaste could be due to compartmentalization of the catalase in peroxisome. Exposure to oxidative stress has been reported to cause a decrease in catalase activity in wheat as well as rice seedlings [44].

Glutathione S-transferases (GSTs) are ubiquitous enzymes that catalyze the conjugation of toxic xenobiotics and oxidatively produced compounds to reduced glutathione, which facilitates their metabolism, sequestration, or removal [10]. The decrease of GST activity in seedlings treated by norflurazon at 10^{-4}M let suggest that this herbicide was not conjugated and / or degraded in the treated seedlings. Although many plant species genes encoding GSTs and showed activities toward colorimetric substrates such as CDNB only a relatively restricted range of plant GSTs showed activities toward herbicides [11].

Pseudomonas putida showed an improvement of the physiological and metabolic parameters of untreated plant seedlings. An increase in photosynthetic pigments and sugars levels could be due to improve mineral nutrition and the growth (data not show) of plants by the bacteria. Nowak [40] observed an increase in chlorophyll content in oregano plants (*Origanum vulgare* L.) inoculated with *Pseudomonas* spp. compared to control plants. Baset Mia et *al*. [5] also showed that PGPR inoculation increased growth parameters such as leaf area, chlorophyll content, and consequently the biomass of banana seedlings (*Musa* sp.) compared to control plants. The increase in crop yields can be attributed to the bacterial growth substances responsible for root system extension and improvement of absorption of mineral elements and water [22]. The mechanism by which PGPR affects the antioxidant responses is not yet clear. A close relationship between plant growth and enzyme activities such as glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), glutathione reductase (GR), and glutathione S-transferase (GST) was demonstrated by [8] in wheat. G6PD, 6PGD, GR, and GST activities were increased at least twice compared with the control by using the effective strains, *Paenibacillus Polymyxa*, *Bacillus megaterium*, and *Pseudomonas putida*. Positive correlations were obtained among these enzymes activities of wheat in values of plant height and leaf area [8]. The observed changes in enzyme activities appeared to be triggered by the PGPR strain selected. Plant GST plays a role in the cellular response to auxins and during the metabolism of plant secondary products [31]. In other hand, Çakmakçı et *al*. [9] showed that the isolated IAA-producing and P-solubilising PGPR strain, , caused the maximum enhancement in GR and GST activities in spinach.

In the presence of norflurazon the isolated rhizobacteria seems to improve the same parameters of stressed seedlings. Membrane damages (lipid peroxidation and leakage of electrolytes) caused by norflurazon were reduced, catalase and GST activities were improved in the presence of the bacteria. This decrease in toxicity norflurazon was inherent to a direct effect of the bacteria on the herbicide metabolism or herbicide chelation by bacterial siderophores or an indirect effect through plant exudation stimulating (synthesis growth substances by the bacteria) and improving mineral nutrition of plants. Our results were in line with those obtained by Munees and Khan [36]. They showed that the PGPR strain *Bradyrhizobium sp.* grew in the presence of high concentrations of selective herbicides quizalafop-P-ethyl and clodinafop had the

ability to enhance the growth of bean seedlings and protect them against the toxic effects of both herbicides. The ability of *Pseudomonas putida* to tolerate strong concentration of the norflurazon might be probably due to the synthesis of auxins (IAA), which directly stimulated the growth and branching of roots thus allowing better absorption of water and nutrients. The EPS provided protection against the drying bacterial cells, phagocytosis and the phages attack [48]. According to Munees and Khan [38], EPS synthesized by different strains of PGPR used to restrict access to seedlings herbicides-treated and thus reduced their toxic effects.

Acknowledgement. I would like to express my deep gratitude to all people who have contributed to the successfulness of this research work.

REFERENCES

[1] Abrous, O., De Paepe, R., Trémolières, A., Mathieu, C., Aïd, F., Benhassaine-Kesri, G., (2009): Evidence that norflurazon affects chloroplast lipid unsaturation in soybean leaves (*Glycine max L.*). Journal of Agricultural and Food Chemistry, 57: 11434-11440.

[2] Ahrens, W.H., (1994): Norflurazon. In Herbicide handbook 7th ed., pp. 218-220. Weed Science Society of America, Champaign, IL.

[3] Ashrafuzzaman, M., Hossen, F.A., Ismail, M.R., Hoque, M.A., Islam, M.Z., Shahidullah, S.M., Meon, S., (2009): Efficiency of plant growth promoting rhizobacteria (PGPR) for the enhancement of rice growth. African Journal of Biotechnology, 8: 1247-1252.

[4] Bajji, M., Kinet, J.M., Lutts, S., (2001): The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regulation, 36 (1): 61-70.

[5] Baset Mia, M.A., Shamsuddin, Z.H., Wahab, Z., Marziah, M., (2010): Effect of plant growth promoting rhizobacterial (PGPR) inoculation on growth and nitrogen incorporation of tissue-cultured Musa plantlets under nitrogen-free hydroponics condition. Australian Journal of Crop Science, 4 (2): 85-90.

[6] Bradford, M.M., (1976): A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72 (1-2): 248-254.

[7] Bramley, P.M., (1994): Carotenoid biosynthesis: a target site for bleaching herbicides. Lipid Biochemistry and Pesticide Action, 22 (2): 525-629.

[8] Çakmakçi, R., Erat, M., Erdogan, Ü. and Dönmez, F., (2007): The influence of plant growth-promoting rhizobacteria on growth and enzyme activities in wheat and spinach plants. Journal of Plant Nutrition and Soil Science, 170: 288-295.

[9] Çakmakçi, R., Erat, M., Oral, B., Erdogan Ü., Şahin, F., (2009): Enzyme activities and growth promotion of spinach by indole-3-acetic acid-producing rhizobacteria. Journal of Horticultural Science & Biotechnology, 84 (4): 375-380.

[10] Dalton, D.A., Boniface, C., Turner, Z., Lindahl, A., Kim, J.H., Jelinek, L., Govindarajulu, M., Finger, R.E., and Taylor, C.G., (2009): Physiological roles of glutathione s-transferases in soybean root nodules. Plant Physiology, 150 (1): 521-530.

[11] Dixon, D.P., McEwen, A.G., Lapthorn, A.J., Edwards, R., (2003): Forced evolution of a herbicide detoxifying glutathione transferase. Journal of Biological Chemistry, 278(3): 23930-23935.

[12] Dorey, S., Baillieul, F., Saindrenan, P., Friting, B., Kauffmann, S., (1998): Tobacco class i and class ii catalase are differentially expressed during elicitor-induced hypersensitive cell death and localized acquired resistance. Molecular Plant-Microbe Interactions, 11: 1102-1109.

[13] Fan, W.J., Zhang, M., Zhang, H.X., Zhang, P., (2012): Improved tolerance to various abiotic stresses in transgenic sweet potato (*Ipomoea batatas*) expressing spinach betaine aldehyde dehydrogenase, PLOS One, 7, e37344.

[14] Fett, W.F., Osman, S.F., Dunn, M.F., (1989): Characterization of exopolysaccharides produced by plant-associated fluorescent pseudomonads. Applied and Environmental Microbiology, 55(3): 579-583.

[15] Goldstein, A.H., Krishnaraj, P.U., (2007): Phosphate solubilizing microorganisms vs. phosphate mobilizing microorganisms: what separates a phenotype from a trait? pp. 203-213. In: Velázquez, E., Rodríguez-Barrueco, C. (eds.), First International Meeting on Microbial Phosphate Solubilization. Springer, Dordrecht, The Netherlands.

[16] Gordon, S.A., Weber, R.P., (1951): Colorimetric Estimation of Indoleacetic Acid. Plant Physiology, 26(1): 192-195.

[17] Habig, W.H., Pabst, M.J., Jacoby, W.B., (1974): Glutathione s-transferases: the first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry, 249(22): 7130-7139.

[18] Hayat, R., Ali, S., Amara, U., Khalid, R., Ahmed, I., (2010) : Soil beneficial bacteria and their role in plant growth promotion: a review. Annals of Microbiology, 60(4): 579-598.

[19] Heath, R.L., Packer, L., (1968): Photoperoxidation in isolated chloroplasts. i. kinetics and stoichiometry of fatty acid peroxidation. Archives of Biochemistry and Biophysics, 125(1): 189-198.

[20] Jung, S., Kim, J.S., Cho, K.Y., Tae, G.S., Kang, B.G., (2000): Antioxidant responses of cucumber (*Cucumis sativus*) to photoinhibition and oxidative stress induced by norflurazon under high and low PPFDs. Plant Science, 153(2): 145-154.

[21] Jung, S., (2004): Expression level of specific isozymes of maize catalase mutants influences other antioxidants on norflurazon-induced oxidative stress. Pesticide Biochemistry and Physiology, 75(1-2): 9-17.

[22] Kennedy, I.R., Tchan, Y.T., (1992): Biological nitrogen fixation in non-leguminous field crops: recent advances. Plant Soil, 141: 93-118.

[23] Kim, C., Apel., K., (2013): $^{1}O_2$-mediated and executer-dependent retrograde plastid-to nucleus signaling in norflurazon-treated seedlings of *Arabidopsis thaliana*. Molecular Plant, 6(5): 1580-1591.

[24] King, E.O., Ward, M.K., Raney, D.E., (1954): Two simple media for the demonstration of pyocianin and fluorescein. Journal of Laboratory and Clinical Medicine, 44(2): 301-307.

[25] Leinho, V., Vacek, O., (1994): Biosynthesis of auxin by phosphate solubilizing rhizobacteria from wheat (*Triticum aestivum*) and rye (*Secale cereale*). Microbiological Research, 149(1): 31-35.

[26] Lemanceau, P., Corberand, T., Gardan, L., Latour, X., Laguerre, G., Boeufgras, J.M., Alabouvette, C., (1995):

Effect of two plant species, flax (*Linum usitatissinum* L.) and tomato (*Lycopersicon esculentum* Mill.), on the diversity of soilborne populations of fluorescent pseudomonads. Applied and Environmental Microbiology, 61(3): 1004-1012.

[27] Lichtenthaler, H.K., (1987): Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods in Enzymology, 148: 350-382.

[28] Lifshitz, R., Kloepper, J.W., Kozlowski, M., Simonson, C., Carlson, J., Tipping, E.M., Zaleska, I., (1987): Growth promotion of canola (rapeseed) seedlings by a strain of *Pseudomonas putida* under gnotobiotic conditions. Canadian Journal of Microbiology, 33(5): 390-395.

[29] Lottmann, J., Heuer, M., De Vries, J., Mahn, A., Düring, K., Wackernagel, W., Smalla, K., Berg, G., (2000): Establishment of introduced antagonistic bacteria in the rhizosphere of transgenic potatoes and their effect on the bacterial community. FEMS Microbiology Ecology, 33(1): 41-49.

[30] Marra, L.M., de Oliveira, S.M., Sousa Soares, C.R.F., de Souza Moreira, F.M., (2011): Solubilisation of inorganic phosphates by inoculant strains from tropical legumes. Scientia Agricola (Piracicaba, Braz.), 68(5): 603-609.

[31] Marrs, K., (1996): The Functions and Regulation of Glutathione S-Transferases in Plants. Annual Review of Plant Biology, 47: 127-158.

[32] Mehnaz, S., Weselowski, B., Aftab, F., Zahid, S., Lazarovits, G., Iqbal, J., (2009): Isolation, characterization, and effect of fluorescent pseudomonads on micropropagated sugarcane. Canadian Journal of Microbiology, 55(8): 1007-1011.

[33] Mc Ready, R.M., Guggoz, J.J., Silvicera, V., Owens, H.S., (1950): Determination of Starch and Amylase in Vegetables. Analytical Chemistry, 22: 1156-1160.

[34] Misko, A.L., Germida, J.J., (2002): Taxonomic and functional diversity of pseudomonads isolated from the roots of field-grown canola. FEMS Microbiology Ecology, 42: 399-407.

[35] Mishra, N.P., Mishra, R.K., Singhal, G.S., (1993): Changes in the activities of antioxidant enzymes during exposure of intact wheat leaves to strong visible light at different temperatures in the presence of different protein synthesis inhibitors, Plant Physiology, 102: 867-880.

[36] Munees, A., Khan, M.S., (2009): Effects of quizalafop-p-ethyl and clodinafop on plant growth promoting activities of rhizobacteria from mustard rhizosphere. Annals of Plant Protection Sciences, 17(1): 175-180.

[37] Munees, A., Khan, M.S., (2011): Response of greengram [*Vigna radiate* (L.) Wilczek] grown in herbicide-amended soil to inoculation with *Bradyrhizobium sp*. (vigna) RM6. Journal of Agricultural Science and Technology,13(20): 1209-1222.

[38] Munees, A., Khan, M.S., (2011): Assessment of pesticide-tolerance and functional diversity of bacterail strains isolated from rhizospheres of different crops. Insight Microbiology, 1(1): 8-19.

[39] Nautiyal, C.S., (1999): An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiology Letters, 170(1): 265-270.

[40] Nowak, J., (1998): Benefits of in vitro ‘‘biotization’’ of plant tissue cultures with microbial inoculants. In Vitro Cellular & Developmental Biology – Plant, 34: 122–130.

[41] Paul, D., Nair, S., (2008): Stress adaptations in a Plant Growth Promoting Rhizobacterium (PGPR) with increasing salinity in the coastal agricultural soils. Journal of Basic Microbiology, 48(5): 378-384.

[42] Patten, C., Glick, B., (1996): Bacterial biosynthesis of indole-3-acetic acid. Canadian Journal of Microbiology, 42(3): 207-220.

[43] Sagar, A.D., Horwitz, B.A., Elliotr, R.C., Thompson, W.F., Briggs, W.R., (1988): Light effects on several chloroplast components in norflurazon-treated pea seedlings. Plant Physiology, 88(2): 340-347.

[44] Shim, I.S., Momose, Y., Yamamoto, A., Kim, D.W., Usui, K., (2003): Inhibition of catalase activity by oxidative stress and its relationship to Salicylic acid accumulation in plants. Plant Growth Regulation, 8: 285-292.

[45] Shuvasish, C., Piyalee, P., Lingaraj, S., Sanjib Kumar, P., (2013) : Reactive oxygen species signaling in plants under abiotic stress. Plant Signaling & Behavior, 8:4, e23681.

[46] Sivasakthivelan, P., Saranraj, P., (2013): *Azospirillum* and its formulations: a review. International Journal of Microbiological Research, 4(3): 275-287.

[47] Sorensen, J., Jensen, L.E., Nybroe, O., (2001): Soil and rhizosphere as habitat for *Pseudomonas* inoculants. Plant and soil, 232: 97-108.

[48] Tank, N., Saraf, M., (2003): Phosphate solubilization, exopolysaccharide production and indole acetic acid secretion by rhizobacteria isolated from *Trigonella foenum-graecum*. Indian Journal of Microbiology, 43: 37-40.

[49] Tsavkelova, E., Cherdyntseva, T., Netrusov, A., (2005): Auxin production by bacteria associated with orchid roots. Microbiology, 74(1): 46-53.

[50] Tsun-Thai, C., Fadzillah, N.M., Kusnan, M., Mahmood, M., (2005): L’effet du stress oxydant sur les cultivars ‘Berangan’ et ‘Mas’. InfoMusa, 14(2): 32-36.

[51] Vincent, J.M., (1970): A manual for the practical study of the root nodule bacteria. International Biological Programme Handbook No.15, Blackwell Science Publications. Oxford, England. 164 p.

[52] Voigt, C., Oster, U., Börnke, F., Jahns, P., Dietz, K.J., Leister, D., and Kleine, T. (2010) : In-depth analysis of the distinctive effects of norflurazon implies that tetrapyrrole biosynthesis, organellar gene expression and ABA cooperate in the GUN-type of plastid signaling. Physiologia Plantarum, 138: 503-519.

Received: 8 May 2018
Accepted: 1 August 2018
Published Online: 7 August 2018
Analele Universităţii din Oradea, Fascicula Biologie
http://www.bioresearch.ro/revistaen.html
Print-ISSN: 1224-5119
e-ISSN: 1844-7589
CD-ISSN: 1842-6433
University of Oradea Publishing House