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EFFECT OF RHIZOBACTERIA INOCULATION AND HUMIC ACID APPLICATION ON CANOLA (*BRASSICA NAPUS* L.) CROP

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Abstract

This study investigated eco-friendly approach of utilizing plant growth promoting rhizobacteria (PGPR) and humic acid (HA) as bio-stimulants to improve the growth, yield and nutrition of canola (*Brassica napus* L.). In this study, we isolated 20 indigenous rhizobacterial strains that were subsequently screened and characterized for their plant growth promoting traits. After that one promising PGPR strain identified as *Acinetobacter pittii* by 16S rRNA gene sequencing was selected for field trial. The field experiment was conducted using RCB design with split-plot arrangement that was replicated four times. Three levels of humic acid (0, 10 and 20 kg ha^{-1}) as main plot factor and two treatments of PGPR (with and without PGPR) as sub-plot factor were used. Data was recorded on plant height (cm), root dry matter $plant^{-1}$, number of lateral root $plant^{-1}$, number of pods $plant^{-1}$, number of seeds pod^{-1}, 1000 seed weight (g), seed yield(kg ha^{-1}), oil content (%), nitrogen (N), phosphorus (P) and potassium (K) contents and uptake. For most of the above mentioned parameters, significant enhancement was observed with the increment of humic acid, and also PGPR treatments were better than their respective control treatments. Maximum values of these parameters were recorded for the interaction of 20 kg HA ha^{-1} with the PGPR strain. It can be concluded that integrated application of HA and PGPR is a better strategy to improve nutrition and yield of canola.

Key words: Rhizobacteria, Canola, Humic acid, Yield Components, Oil Content.

Introduction

Production of enough food to ensure the food security of rapidly increasing population especially in arid regions is becoming challenging day by day. This is because these areas are characterized with various types of environmental stresses that badly hamper their crop production (Lioubimtseva & Henebry, 2009). Furthermore, yield of crops is stagnant even with the use of chemical fertilizers due to low fertilizer use efficiency (FUE). The use of mineral fertilizers is inevitable to fulfill food requirements of rapidly growing world population with limited land resources (Bindraban *et al.,* 2015). Unfortunately FUE is usually very low due to various environmental and soil factors/processes (Simpson *et al.,* 2011). Most part of the applied fertilizers is not available to the plants and is subjected to losses by leaching, erosion, volatilization, fixation, runoff, denitrification, precipitation etc. (Gourley *et al.,* 2012). This low FUE is not only causing economic losses but also imposing serious water and environmental pollution problems (Dibb *et al.,* 2003). As fertilizers are considered best source of readily available essential plant nutrients so farmer think that increasing use of fertilizer will increase the crop yield. This trend of increasing fertilizer consumption promotes excessive application of fertilizer which enhances the chances of gaseous losses of fertilizer to atmosphere, movement of nitrates to underground water and water bodies causing eutrophication (Wu & Ma, 2015).

The present scenario warrants the scientific community to find some sustainable solutions to increase the fertilizer use efficiency and decrease the nutrients losses. Various approaches have been used to enhance FUE, but the use of plant biostimulants like HA and PGPR in this regard have been documented as potential and sustainable tools to overcome this problem. Plant biostimulants are actually substances and/or microorganisms which can enhance the nutrients use efficiency by stimulating the natural processes like nutrient uptake and translocation, nutrient utilization, better root growth and physiological and metabolic processes (Calvo *et al*., 2014).

Humic acids are recognized as dark colored organic substances which have been reported to enhance nutrient availability, nutrient absorption, nutrient utilization, plant growth, physiology and metabolism through various mechanisms (Schiavon *et al.,* 2010; Berbara & Garcia, 2014). Because of the unique structural characteristics of humic acids with large number of oxygen containing functional groups (CO_2H_2, OH, phenols, and C=O), they play a key role to enhance nutrient availability by the chelation of metallic nutrient elements (Nardi *et al.,* 2009). Better root architecture and increased root area by stimulating root elongation and lateral root development have been reported as important phenomena responsible for improved nutrient uptake by humic acids (Canellas *et al.,* 2002, 2011; Jindo *et al.,* 2012).

Plant Growth Promoting Rhizobacteria (PGPR) are the free living and/or associative soil bacteria which habitat in the close vicinity of plant roots and interact with plants through different ways. Mechanisms through which PGPR can improve nutrient use efficiency are: enhanced availability and uptake of nutrients by solubilization of nutrients through organic acids secretion; improved nutrient uptake through better root growth; sequestration and uptake of iron (Fe) by the production of siderophores (Adesemoye *et al.,* 2010; Khan *et al.,* 2009; Sharma *et al.,* 2013). In addition PGPR can also promote plant growth and yield by producing plant growth regulator (PGRs) such as auxins, gibberellin and cytokinins etc. (Bhattacharyya & Jha, 2012; Kang *et al.,* 2012; Afzal *et al.,* 2015). Furthermore, both PGPR and HA have also

been reported to alleviate the negative impacts of biotic and abiotic stresses due to their positive role in plant physiology and metabolism and by various other mechanisms (Shrivastava & Kumar, 2015).

Previously, researchers (Shahzad *et al.,* 2103; Mehta *et al.,* 2015; Habibi *et al.,* 2014; Zhahid *et al.,* 2015) have confirmed the promising effects of different PGPR species on various crops in laboratory and field experiments under variable ecological conditions. However, soil-plant-microbes interactions have been documented as complex phenomena and PGPR vary widely from region to region due to variability in ecological conditions (Lucy *et al.,* 2004). Thus, it is important to explore and identify region specific PGPR strains—as they will best fitting for enhancing crop growth and yield in that region (Verma *et al.,* 2013).

Previously, there was no work done in the study area regarding PGPR, so significance of indigenous PGPR in promoting crop production prompted us to explore the potential of locally isolated PGPR which might perform better due to being adaptable to local climatic conditions. Moreover, individual effects of both humic acids and PGPR have been investigated by researchers on canola and various other crops. However, there is very scarce work reported on the integrated role of humic acid and PGPR on nutrition aspects of canola. As humic acid also has positive effect on PGPR so we hypothesized that interaction of both might produce more promising results.

Materials and Methods

Bacterial isolation and purification: For the isolation of PGPR, soil samples from rhizosphere of five different plants were taken from Agriculture Research Station of King Abdulaziz University situated at Hada Al Sham (21o 48' 3" N, 39o 43' 25"E) having altitude 235 m above sea level and arid climatic conditions (Fig. 1). A composite sample of different sampling location was also made and analyzed for pH (7.82), electrical conductivity (3.45 dS m^{-1}) and organic matter contents (0.65%). Bacterial isolation was carried out by dilution plate technique with phosphate buffered saline (PBS) using Luria Bertani (LB) agar media following the protocol described by Baig *et al.* (2012). Briefly, soil suspension (soil: PBS ratio 1:10 w/v) was prepared in sterilized PBS and a loopful of the suspension was streaked on solidified LB agar plates and plates were incubated at 28^{o}C until the appearance of bacterial colonies. After that 20 morphologically different colonies (depending on colony shape, appearance, margin and color) were isolated and purified by sub-culturing on fresh agar plates every time. Then purified single colonies were stored in 35% glycerol (w/v) at -80^{o}C for further evaluation.

Screening of PGPR on the basis of their growth promoting potential: To find out most promising PGPR strain among the 20 isolated bacterial strains a screening trial in growth chamber with four replications of each strain using peat moss as growth medium was conducted. Broth culture of each strain in tryptic soy broth (TSB), a general purpose medium was prepared. For that, single colony of each strain was transferred to conical flask containing 250 ml sterilized TSB and flasks were put in shaking incubator (100 rpm) at 28^{o}C for 3 days. Before inoculation, the seeds of canola (*Brassica napus* L.) (cv. UAF-11) were surface disinfected first by keeping in 95% for short time and then by dipping in 0.2% $HgCl_2$ for three minutes. After that washing of seeds was done with sterile water and they dipped in sterilized water to imbibe. Then imbibed seeds were placed in broth culture for 1 hour to inoculate with PGPR. Control seeds were treated with sterilized TSB solution. Subsequently the seeds were allowed to grow in jiffy-7 plates for 3 weeks. Sterile Hoagland nutrient solution was used to irrigate the seedlings. Plates were placed in growth chamber and after 3 weeks, shoot length (cm), shoot fresh weight (g $plant^{-1}$), root length (cm) and root fresh weight (g $plant^{-1}$) were recorded and three most promising strains were selected by keeping in view their positive effect on above mentioned plant growth parameters for further characterization and field evaluation.

Biochemical characterization of PGPR to assess their growth promoting traits: The three most promising PGPR strains selected through screening were assessed for their growth promoting traits such as auxins (IAA) production potential, phosphate solubilization, ACC deaminase production potential, siderophores production and phosphatase production. For all these characterization, standard procedures were followed as described below. Gram staining of the selected strains was also performed according to protocol described by Holt *et al.* (1994).

IAA production: The IAA production potential of selected strains was assessed with and without tryptophan (a precursor of IAA) through protocol proposed by Gordon & Weber (1951). According to protocol, fresh culture of each selected bacterial strain was prepared by inoculating the sterilized TSB and incubating it at 28±2^{o}C for 48 hours. Then 5 mL of LB broth supplemented with and without 500 µg mL^{-1} tryptophan, was inoculated with 100 µL culture of each strain in triplicate. These cultures were placed under constant shaking at 28±2^{o}C for 7 days. After one week of growth, cell free supernatant was obtained through centrifugation at 10,000 rpm for 10 minutes. To determine the IAA concentration, 2 ml of supernatant was taken and two drops of orthophosphoric acid and 4 ml of Salkowski's reagent (1 ml 0.5M $FeCl_3$ in 50 ml 35% perchloric acid) were added in it. The mixture was kept for 15 minutes to develop a pink color (pink color indicates the presence of IAA). Then for quantitative measurement of IAA, optical density of the mixture was recorded by spectrophotometer at 530 nm. Finally, concentration of IAA in the mixture was determined with the help of standard curve developed by using working standards of pure indole-3-acetic acid.

P solubilization: Quantitative determination of the P solubilization activity of selected strains was carried out by using phospho-molybdate blue color method as described by Hayat *et al.* (2013). Briefly, 100 ml of sterilized Pikovskaya (Pikovskaya, 1948) broth with initial pH of 7 including 5 g/L of tricalcium phosphate (as insoluble

phosphate) was inoculated with 100 µL of bacterial culture prepared in TSB. Inoculated broth was placed in shaking incubator at 100 rpm and 28±2ºC for 7 days. After 7 days of incubation, pH of the medium was recorded and cell free supernatant was obtained through centrifugation at 8500 rpm for 25 minutes. Quantitative measurement of soluble P in the supernatant was carried out according to phosphor-molybdate blue color method by recording absorbance with spectrophotometer at 882 nm using working standards of KH_2PO_4 solutions for standard curve.

ACC-deaminase activity: For the quantitative measurement of ACC-deaminase activity, protocol described by Honma & Shimomura (1978) was employed. Accordingly, concentration of α-ketobutyrate in the mixture produced by the breakdown of ACC was measured at 540 nm by comparing with standard curve drawn by taking the absorbance of various levels of pure α-ketobutyrate standards (0.1 to 1.0 µmol).

Siderophore production: The selected PGPR strains were also assessed for their ability to produce siderophore on solidified agar plates of chrome azurole S (CAS) media according to Schwyn & Neilands (1987). For this purpose fresh culture of bacterial strains were spot inoculated on agar plates and plates were kept in incubator for five days at 30ºC. The appearance of a yellow orange halo indicated that strain has potential for siderophore production.

Phosphatase activity: Phosphatase activity of selected PGPR strains was determined qualitatively on TSA agar plates (having 2 ml of 0.5% phenolphthalein diphosphate solution per 100 ml media) by following the procedure adopted by Ribeiro & Cardoso (2012). Solidified TSA plates were spot inoculated with fresh culture of selected strains by inoculating needle and plates were kept in incubator at 28ºC for 2 days. Presence of phosphatase production potential was confirmed by the formation of pink zone around the bacterial colonies.

Identification of selected rhizobacterial strain through 16S rRNA gene sequencing: For the identification of finally selected PGPR strain, 16S rRNA technique was used according to protocol described by Yasir *et al.* (2009). Extraction of genomic DNA of bacteria was done by using 5% Chelex-100 solution and boiled for 20 min in 1.5 mL tubes. Then polymerase chain reaction (PCR) was performed using supernatant as template. Universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used for PCR amplification of 16SrRNA gene. The temperature conditions during amplification process were; 94ºC for 5 minutes (for 1 cycle); 94ºC for 45 seconds, 55ºC for 45 seconds and 72ºC for 1 minutes (for 30 cycles); and 72ºC for 10 minutes (for 1 cycle). After amplification, purified PCR product was sequenced through Sanger sequencing technology, using ABI prism sequencer 3730 (Applied Biosystems, USA) according to the protocol described by manufacturer. Then EzTaxon server (http://eztaxon-e.ezbiocloud.net/) was used for blasting to identify the related type strain. The sequence was aligned using clustalX and a phylogenetic tree (Fig. 2) was designed by distance method (neighbour-joining) using MEGA6 software (Tamura *et al.*, 2013).

Field trial: To investigate the integrated effect of indigenous PGPR and humic acid on the growth, yield and nutrition of canola, a 2-year field trial was executed at Agriculture Research Station of King Abdulaziz University situated at Hada Al Sham (21o 48' 3" N, 39o 43' 25"E). The average temperature and rainfall during growth period are given in Fig. 2. Prior to experiment, field soil was analyzed (Table 1) by following the procedures of Ryan *et al.* (2001). Experiment was planned according to RCBD-split plot design with four replications. Three levels of HA (HA_0= 0 kg ha^{-1}, HA_{10}= 10 kg ha^{-1} and HA_{20}= 20 kg ha^{-1}) as main plot factor and two levels of PGPR (P_0= without PGPR and P_1= with PGPR) as sub-plot factor were applied according to the experimental design after plowing of the field. PGPR was applied as seed inoculation. The inoculation of canola seeds (cv. UAF-11) was done after surface sterilization (as described earlier). After surface disinfection, inoculation of seeds was carried out by a mixture containing broth culture of selected strain, 10 % sterilized solution of sugar and peat and kaolin (clay). Control (uninoculated) seeds were coated with the mixture containing all the components without bacteria. Sowing of the crop was done in lines by maintaining a row to row spacing of 40 cm in sub plots of 2 x 2.5 m^2 dimension.

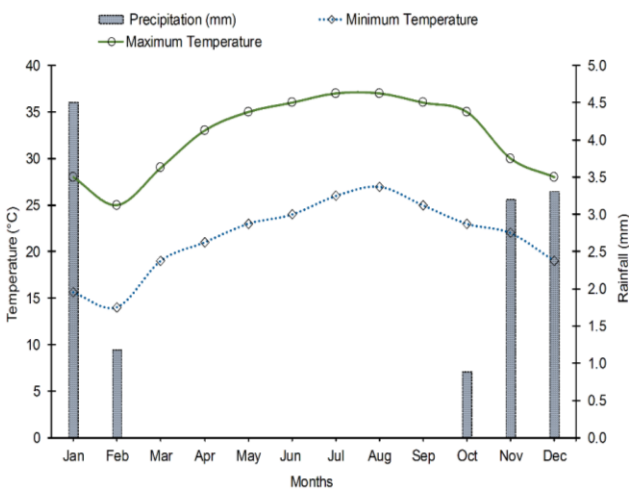

Fig. 1. Climatic conditions of the experimental area.

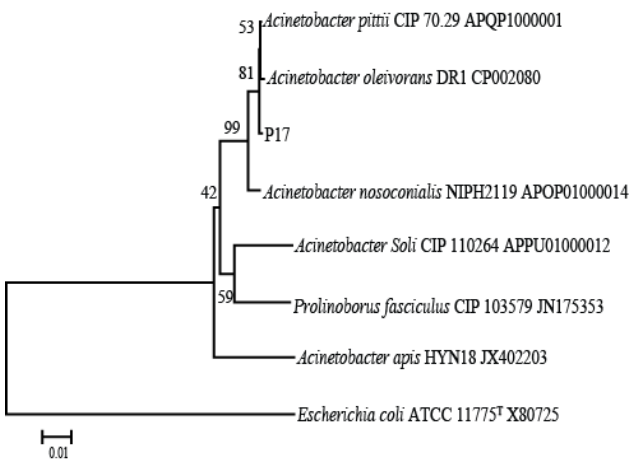

Fig. 2. Phylogenetic tree constructed by neighbor joining method showing relationship of P17 with closely related species.

Table 1. Characteristics of soil used for field experiment.

Characteristics	Unit	Value
Texture		loamy sand
pH		7.68
EC	dS m^{-1}	3.24
OM	%	0.65
Total N	%	0.054
Available P	mg kg^{-1}	6.4
Extractable K	mg kg^{-1}	355
Fe	mg kg^{-1}	54
Zn	mg kg^{-1}	17
Mn	mg kg^{-1}	2
Cu	mg kg^{-1}	5

Data collection and plant analysis: Data on plant height (cm), root dry matter (g/plant), no. of lateral roots (per plant), pods per plant, seeds per pod, thousand seed weight (g), and seed yield (Kg ha^{-1}), was recorded at harvesting following similar procedure as described by Daur *et al*., 2011; Ihsan *et al*., 2016. Data on root dry matter and number of lateral roots was recorded by uprooting the plants at pod formation stage. At the time of harvesting, 10 plants from each sub plot were randomly selected at tagged to record data on plant height (cm), pods per plant and seeds per pod. For seed yield, 4 central rows in each sub plot were harvested. For the determination of NPK contents in canola seeds, the ground and oven dried seeds of canola were digested with H_2O_2 and H_2SO_4 following the protocol described by Ahmad *et al*. (2014)**.** The N in digested filtrate was measured by Kjeldahl method and P and K by spectrophotometer and inductively coupled plasma-optical emission spectrometer (ICP-OES, Varian 720-ES, Palo Alto, USA) respectively. Seed oil contents were determined by accelerated solvent extraction method (Matthäus & Brühl, 2001) using petroleum ether as solvent. Following formula was used to calculate NPK uptake in canola seed:

NPK uptake in seed = [% NPK in seed/100] × grain yield (kg/ha)

Statistical analysis: The data recorded for each trait was separately analyzed using Statistic 8.1 software and the means were compared with the help of LSD test according to Steel and Torrie (1960).

Results

Isolation, purification and screening of PGPR: Initially, 20 different bacterial colonies were isolated on the basis of morphological features (Table 2) like colony shape, appearance, margins, and color. Clear variations in morphological features were observed among the isolates. Out of 20 bacterial colonies 14 were observed with round shape while 6 colonies were of irregular shape. With respect to appearance, 10 isolates were categorized as shiny, 5 as dull and 5 as intermediate. The isolates were also different in their morphology of colony margins, where 8 isolates were having wavy margins while all the rest were with smooth margins. Moreover, different colony colors including white, off white, yellow, pinkish and green were observed for the isolates. Keeping in view the above morphological features, the isolates were purified by serial restreaking on fresh agar plates each time and named as SH-1 to SH-20. To find the most promising PGPR strains for plant growth promoting, a screening trial was conducted and data regarding shoot and root lengths (cm) and shoot and root fresh weights (g $plant^{-1}$) was recorded. The data (Table 3) indicated that three isolates (SH-4, SH-9 and SH-17) were more promising compared to control and other isolates. Maximum value recorded for shoot length was 28 cm that was observed for SH-9 and SH-17 followed by 27 cm for SH-4 while minimum was 20 cm for control, SH-8, SH-10, and SH-12. The increase in the shoot length by SH-4, SH-9, and SH-17 over the control was 35, 40 and 35% respectively. Thus, rest of the PGPR shoot length ranged between 20—28 cm. Shoot fresh weight (g $plant^{-1}$) was also statistically superior for SH-9 and SH-17 compared to control and all other isolates. The increase in shoot fresh weight was 40 and 41% for SH-9 and SH-17 respectively over the control treatment. Moreover, root length (cm) of SH-4 and SH-17 outscored over control by 38 and 42% respectively. The results for root fresh weight (g $plant^{-1}$) indicated that highest root fresh weight (1.29 g) was recorded with SH-17 which was 53% higher than control. The root fresh weight subsequently reduced to 1.24 and 1.20 g in SH-4, and SH-9 that correspond for 47 and 42% increase over control. Thus, on the basis of results of this screening trial, we selected 3 most promising isolates (SH-4, SH-9 and SH-17).

Characterization of PGPR for plant growth promoting traits: Results of biochemical characterization of selected isolates demonstrated that all the selected strains exhibited variations in their plant growth promoting characteristics (Table 4). Isolate SH-17 showed more potential of IAA production which produced 5.12 and 2.34 µg IAA mL^{-1} with and without tryptophan respectively. Likewise, SH-4 and SH-9 produced 3.73 and 3.41 µg IAA mL^{-1} with and 1.79 and 1.24 µg IAA mL−1 without tryptophan respectively. P solubilization potential of selected isolates was evaluates by measuring the amount of P (mg P mL^{-1}) in Pikovskaya broth having 5 g/L of tricalcium phosphate (as insoluble phosphate). Maximum soluble P (39.87 mg/mL) was recorded in the media inoculated with SH-17 followed by SH-9 (28.57 mg/mL) and SH-4 (19.15 mg/mL). Maximum pH (5.24) was recorded in the media inoculated with SH-4 followed by SH-9 (5.11) and SH-17 (4.89), thus we found pH of the media in an inverse relation with P solubility. Like other traits, ACC-deaminase activity (production of α-ketobutyrate with the action of ACC-deaminase) was also variable in selected isolates. The isolate SH-17 produced 197 µM α-ketobutyrate mg^{-1} h^{-1} that exceeded the two other strains in this trait, as SH-4 produced 15 µM α-ketobutyrate mg^{-1} h^{-1} and SH-9 was not found to possess ACC-deaminase activity. Besides, SH-17 was also able to synthesize siderophore, whereas the other two isolates (SH-4 and SH-9) did not show the ability of siderophore production. Moreover, all the three strains (SH-4, SH-9, and SH-17) were found positive for phosphatase activity. Keeping in view the biochemical characterization, SH-17 showed the greatest potential compared with others, thus we selected this strain for identification and field evaluation.

Table 2. Morphological features of bacterial isolates observed during preliminary selection.

Strain code	Colony shape	Appearance	Margin	Color
SH-1	Round	Shiny	Smooth	Whitish
SH-2	Irregular	Dull	Smooth	Off whitish
SH-3	Irregular	Dull	Smooth	Whitish
SH-4	Round	Dull	Smooth	Yellowish
SH-5	Round	Intermediate	Smooth	Yellowish
SH-6	Round	Dull	Smooth	Yellowish
SH-7	Round	Shiny	Smooth	Whitish
SH-8	Round	Shiny	Smooth	Whitish
SH-9	Round	Intermediate	Smooth	Yellowish
SH-10	Round	Shiny	Smooth	Off whitish
SH-11	Round	Shiny	Wavey	Light green
SH-12	Irregular	Intermediate	Wavey	Light green
SH-13	Round	Intermediate	Wavey	Off whitish
SH-14	Irregular	Dull	Wavey	Pinkish
SH-15	Irregular	Shiny	Wavey	Pinkish
SH-16	Round	Shiny	Wavey	Off whitish
SH-17	Irregular	Intermediate	Wavey	Off whitish
SH-18	Round	Shiny	Smooth	Off whitish
SH-19	Round	Shiny	Smooth	Off whitish
SH-20	Round	Shiny	Wavey	Yellowish

Table 3. Effect of PGPR inoculation on growth parameters of canola under growth chamber experiment.

Strain code	Shoot length (cm)	Shoot fresh weight (g/plant)	Root length (cm)	Root fresh weight (g/plant)
Control	$20^{d\,\dagger}$ (0)‡	1.50^{g} (0)	6^{e} (0)	0.85^{i} (0)
SH-1	22^{c} (11)	1.55^{g} (3)	7^{cd} (17)	0.86^{hi} (2)
SH-2	24^{bc} (19)	1.88^{b} (25)	7^{cd} (17)	1.04^{d} (23)
SH-3	25^{abc} (25)	1.64^{ef} (9)	6.25^{de} (4)	1.06^{d} (26)
SH-4	27^{ab} (35)	1.89^{b} (26)	8.25^{a} (38)	1.24^{ab} (47)
SH-5	23^{cd} (15)	1.55^{g} (3)	6.25^{de} (4)	0.85^{i} (1)
SH-6	23^{cd} (13)	1.84^{b} (23)	6.5^{cde} (8)	1.03^{de} (22)
SH-7	25^{abc} (25)	1.61^{f} (7)	7^{cd} (17)	1.06^{d} (26)
SH-8	20^{d} (0)	1.61^{f} (7)	6.25^{de} (4)	0.98^{ef} (16)
SH-9	28^{a} (40)	2.10^{a} (40)	8^{ab} (33)	1.20^{b} (42)
SH-10	20^{d} (0)	1.67^{de} (11)	6.25^{de} (4)	0.91^{gh} (7)
SH-11	25^{abc} (25)	1.51^{g} (1)	7.25^{bc} (21)	0.95^{fg} (13)
SH-12	20^{d} (0)	1.55^{g} (3)	7.25^{bc} (21)	1.05^{d} (25)
SH-13	24^{bc} (18)	1.67^{de} (11)	7^{cd} (17)	1.11^{c} (32)
SH-14	22^{cd} (10)	1.50^{g} (0)	6^{e} (0)	0.96^{f} (14)
SH-15	24^{bc} (21)	1.75^{c} (16)	6.25^{de} (4)	0.88^{hi} (4)
SH-16	24^{bc} (20)	1.87^{b} (24)	6.25^{de} (4)	0.96^{f} (14)
SH-17	28^{a} (40)	2.12^{a} (41)	8.5^{a} (42)	1.29^{a} (53)
SH-18	23^{cd} (15)	1.89^{b} (26)	6.75^{cde} (13)	1.07^{cd} (26)
SH-19	22^{cd} (11)	1.71^{cd} (14)	6.25^{de} (4)	0.97^{f} (15)
SH-20	24^{c} (19)	1.85^{b} (23)	6.75^{cde} (13)	0.89^{hi} (6)

† Values followed by different letter (s) are statistically different at $p<0.05$ according to LSD test. ‡ Values given in the parenthesis ( ) are representing the % increase over control

Identification of the finally selected PGPR strain: The results of 16S rRNA sequencing analysis of selected strain SH-17 indicated as P17 in Fig. 2 exhibited highest 16S rRNA gene sequence similarity values with *Acinetobacter pittii* CIP 70.29^T (99.9%) followed by *Acinetobacter oleivorans* DR1^T (99.6%) and *Acinetobacter nosocomialis* NIPH 2119^T (99.2%). In the phylogenetic tree, P17 strain joined the cluster comprising the *Acinetobacter pittii* and *Acinetobacter nosocomialis* species with 99% bootstrap support.

Effect of HA and PGPR on growth and yield of canola under field conditions: The results of integrated effect of HA and PGPR (*Acinetobacter pittii*) on growth and yield parameters under field conditions is presented in Table 5. The results clearly demonstrated that all the studied parameters were significantly improved by humic acid and PGPR application. However, interaction of HA and PGPR was found significant only for root dry matter, number of lateral roots and seed yield.

Mean comparison between different rates of HA (0, 10 and 20 kg HA ha^{-1}) for their effect on plant height showed that application of HA @ 20 kg ha^{-1} produced maximum plant height (127 cm) that was significantly ($p\leq0.05$) higher compared to plots that received 0 kg HA ha^{-1}. Plant height in plots that received 10 kg HA ha^{-1} (114 cm) was statistically non-significant with 0 kg HA ha^{-1} (105 cm) plots. Similarly, application of PGPR (P_1 = *Acinetobacter pittii*) significantly ($p\leq0.05$) induced more plant height (119 cm) than the uninoculated treatment (112 cm).

Root dry matter (g plant^{-1}) and number of lateral roots per plant were also remarkably improved by application of HA and PGPR. Also, significant interaction was observed between HA and PGPR in their effect on root dry matter and number of lateral roots. Maximum root dry matter (20.33 g) and number of lateral roots plant^{-1} (39) were for HA_{20} while minimum root dry matter (11.72 g) and number of lateral roots plant^{-1} (27) were for HA_0. Interaction of HA and PGPR on root dry matter and number of lateral roots is presented in Figs. 3 and 4 respectively which revealed that interaction comprising 20 kg HA ha^{-1} x PGPR was statistically better than all other interactions. Maximum root dry matter (24.23 g) and number of lateral roots (45) were recorded for the HA_{20} x P_1 interaction while, minimum root dry matter (10.54 g) and number of lateral roots (26) were observed for HA_0 x P_0 interaction.

Number of pods plant^{-1} and number seeds pod^{-1} indicated patterns for HA and PGPR similar to those trends which were observed earlier for plant height. More pods (172) were observed for 20 kg HA ha^{-1} followed by 156 and 141 pods plant^{-1} for 10 and 0 kg HA ha^{-1} respectively. PGPR inoculation also showed promising effects on number of pods and produced significantly higher (163) pods than the treatment without PGPR. Likewise, HA application @ 20 kg ha^{-1} produced 28 seeds pod^{-1} followed 24 seeds pod^{-1} that was recorded for 10 kg HA ha^{-1} application. Least number of seeds pod^{-1} (21) was recorded where no humic acid was applied. The positive effect of PGPR on number of seeds pod^{-1} was confirmed by registering significantly ($p\leq0.05$) higher number of seeds (26) in PGPR treated plots compared to 23 seeds pod^{-1} in plots without PGPR applications.

Data regarding the effect of HA and PGPR on 1000 seed weight of canola indicated that both humic acid and PGPR have significant effect on this parameter. 1000 seed weight increased with the increment of HA level, consequently 2.36, 3.16 and 3.73 g 1000 seed weights was recorded for 0, 10, and 20 kg HA ha^{-1} respectively. Application of *Acinetobacter pittii* also showed positive effects on 1000 seed weight of canola by significantly improving this parameter. Maximum 1000 seed weight of 3.29 g was recorded in plots with PGPR compared to 2.88 g without PGPR application.

The crop total seed yield is closely related to yield components, so like yield components (pods plant^{-1}, number of seeds pod^{-1}, and 1000 seed weight) HA and PGPR application also showed significant effect on this parameter. For seed yield, significant ($p\leq0.05$) interaction between HA and PGPR was observed (Fig. 5). The results presented in figure clearly indicated that application of PGPR at each level of humic acid has produced more seed yield than without PGPR at the same level of humic acid. Maximum seed yield (1481 kg ha^{-1}) was achieved with the combined application of PGPR and HA @ 20 kg ha^{-1}, which is significantly higher than all other interactions. Minimum seed yield (1001 kg ha^{-1}) was observed where no PGPR and HA was applied.

In this study significant ($p\leq0.05$) improvement in the oil contents of canola seed was observed with humic acid, while application of PGPR (*Acinetobacter pittii*) slightly improved oil content compared to no PGPR. Maximum oil contents (43.20%) were recorded where HA was applied @ 20 kg ha^{-1} that was significantly higher than the oil contents (40.83%) produced under no HA application. However, the oil content of canola under 20 kg HA ha^{-1} application was statistically similar to that produced under 10 kg HA ha^{-1} application (41.95%).

Effect of HA and PGPR on NPK concentration and uptake under field conditions: The analyzed data regarding the combined effect of HA and PGPR on NPK contents and uptake in canola seed is presented in table (Table 6). Trend of the data indicated that application of HA and PGPR significantly improved NPK concentrations and uptake in the canola seed. N content (%) of the seed was maximum (4.49%) in plots where HA was applied @ 20 kg ha^{-1} while it was minimum (3.86%) in plots that received 0 kg HA ha^{-1}. Although application of 20 kg HA ha^{-1} prove to induce a little high N content (4.49%) in the seed compared to 10 kg HA ha^{-1} (4.16%) but statistically both values were comparable. Similarly, PGPR application with 4.31% N in the seed was also statistically superior to no PGPR application.

P concentration in canola seed significantly ($p\leq0.05$) improved by HA and PGPR independently, and by their interaction. The increasing trend in P contents of seed was noted with increasing rate of humic acid application, thus maximum and minimum P contents were recorded as 0.85 and 0.62 % for 20 and 0 kg HA ha^{-1} respectively. Also, P content of seed for PGPR treatments (0.76 %) was noticed higher than without-PGPR treatments. Mean comparison of different interactions (Fig. 6) revealed that maximum seed P content (0.94%) was observed for 20 kg ha^{-1} humic acid x P_1 interaction while minimum P content (0.61 %) was recorded for 0 kg HA ha^{-1} x P_0 interaction.

Table 4. Results of characterization for the selected bacterial strains.

Strain	IAA production (µg mL^{-1})		P solubilization (mg P mL^{-1})		ACC-deaminase activity (µM α-ketobutyrate mg^{-1} h^{-1})	Siderophore production	Phosphatase production	Gram reaction
	With tryptophan	Without tryptophan	P in medium	pH of Medium				
SH-4	3.73	1.79	19.15	5.24	15	-	+	+ve
SH-9	3.41	1.24	28.57	5.11	-	-	+	-ve
SH-17	5.12	2.34	39.87	4.89	197	+	+	-ve

Table 5. Effect of humic acid and PGPR on growth and yield parameters of canola.

Treatments	Plant height (cm)	Root dry matter (g/plant)	Number of lateral roots (per plant)	No. of pods (per plant)	No. of seeds (per pod)	1000-seed weight (g)	Seed yield (kg/ha)	Oil contents (%)
	Humic acid (kg/ha)							
HA_0 (0 kg)	105^{b}	11.72^{b}	27^{b}	141^{b}	21^{b}	2.36^{c}	1003^{b}	40.83^{b}
HA_{10} (10 kg)	114^{ab}	13.76^{b}	31^{b}	156^{ab}	24^{b}	3.16^{b}	1137^{b}	41.95^{ab}
HA_{20} (20 kg)	127^{a}	20.33^{a}	39^{a}	172^{a}	28^{a}	3.73^{a}	1360^{a}	43.20^{a}
	PGPR (*Acineobacter pittii*)							
P_0 (No PGPR)	112^{b}	13.40^{b}	29^{b}	149^{b}	23^{b}	2.88^{b}	1109^{b}	41.61^{a}
P_1 (With PGPR)	119^{a}	17.14^{a}	35^{a}	163^{a}	26^{a}	3.29^{a}	1224^{a}	42.38^{a}
Significance								
Humic acid	*	**	**	*	**	**	**	*
PGPR	*	**	**	*	*	**	**	ns
HA * PGPR	ns	**	*	ns	ns	ns	*	ns

Values followed by different letter (s) are statistically different
Whereas * and ** show significant difference at 0.05 and 0.01 probability respectively, and 'ns' shows no significant difference

Table 6. Effect of humic acid and PGPR on NPK concentrations and uptake in canola seed.

Treatments	Seed N contents (%)	Seed P contents (%)	Seed K contents (%)	N uptake in seed (kg/ha)	P uptake in seed (kg/ha)	K uptake in seed (kg/ha)
	Humic acid (kg/ha)					
HA_0 (0 kg)	3.86^{b}	0.62^{b}	0.84^{b}	39^{c}	6.21^{c}	8.37^{c}
HA_{10} (10 kg)	4.16^{ab}	0.69^{b}	0.92^{ab}	48^{b}	7.88^{b}	10.43^{b}
HA_{20} (20 kg)	4.49^{a}	0.85^{a}	0.99^{a}	61^{a}	11.65^{a}	13.48^{a}
	PGPR (*Acinetobacter pittii*)					
P_0 (No PGPR)	4.02^{b}	0.68^{b}	0.88^{b}	45^{b}	7.57^{b}	9.79^{b}
P_1 (With PGPR)	4.31^{a}	0.76^{a}	0.95^{a}	54^{a}	9.59^{a}	11.73^{a}
Significance						
Humic acid	**	**	*	**	**	**
PGPR	*	**	**	**	**	**
HA * PGPR	ns	*	ns	**	**	*

Values followed by different letter (s) are statistically different
Whereas * and ** show significant difference at 0.05 and 0.01 probability respectively, and 'ns' shows no significant difference

The concentration of K in the canola seed was also significantly increased by both HA and PGPR but their interaction was non-significant. Mean comparison for seed K contents between different levels of humic acid demonstrated that humic acid @ 20 kg ha^{-1} showed more pronounced effect on seed K contents by accumulating 0.99% K in seeds. Where as treatments with 0 and 10 kg HA/ha were found to have 0.84% and 0.92% K in the seed respectively. Likewise, a higher K content of seed (0.95 %) in P_1 plots was recorded compared to P_0 (0.88%).

N, P and K uptake (kg ha^{-1}) in canola seed in response to HA and PGPR application showed similar pattern and interaction between HA and PGPR was found significant for these parameters. Mean comparison of different interactions for N, P, and K uptake in canola seed are presented in Figs. 7, 8 and 9 respectively. It was noted that interaction of HA_{20} x P_1 attained maximum values for N (69), P (13.90), and K (15.27) uptake (kg ha^{-1}) compared to all other interactions.

Discussion

Isolation, purification and screening of PGPR: In this study we found morphological variations in colony shape, appearance, margin, and color. Variations in morphological features of soil bacteria has also been found earlier and it is reported that these variations could be due to genetic diversity (Kim *et al.,* 2011; Rameshkumar *et al.,* 2012). Existence of morphological variations in rhizospheric bacteria has also been confirmed by previous researchers (Hayat *et al.,* 2013; Habibi *et al.,* 2014; Zahid *et al.,* 2015). Habibi *et al*., (2014) also documented morphological variability in colony form, color, elevations and margins of 166 bacterial isolates obtained from rhizospheric soils of rice, wheat, oats, crabgrass, maize, ryegrass and sweet potato. They found that maximum proportion of bacteria from each rhizosphere soil was whitish and yellowish in color with circular form, raised elevation and entire margins. Likewise, round shape, smooth margins, and off white color were dominant morphological features of rhizobacteria isolated from wheat rhizosphere by Zahid *et al.,* (2015).

The significant improvement in growth parameters (shoot length, shoot weight, root length, root weight) of canola with bacterial inoculation in screening trial conducted under growth chamber conditions served as strong evidence to designate the isolated bacteria as PGPR. The enhancement in growth can be attributed to the ability of PGPR to produce plant growth regulators (PGRs) like IAA, gibberellins, ABA etc. (Belimov *et al.,* 2015; Mahmood *et al*., 2016), as these PGRs play a vital role in cell elongation, cell division, root elongation which ultimately improve plant growth (Campanoni & Nick, 2005; Kiani *et al.,* 2016). Exhibition of IAA production ability by selected strains (SH-4, SH-9 and SH-17) in this study further justified them as PGPR. Furthermore, our results are supported by the findings of Shahzad *et al.* (2013), and Habibi *et al.* (2014), as they have reported simultaneous production of IAA and ACC-deaminase activities for PGPR and parallel improvement in growth parameters (shoot length, root length, shoot weight, root weight and number of lateral roots) under their trials. Similarly, Baig *et al.,* (2014) recorded 89% increase in shoot length, 77% in shoot dry matter, 78% in root length and 57% in root dry matter in a 3 week axenic jar trial on maize using *Bacillus* sp. Cp-h60 possessing IAA production potential, P solubilization ability and ACC-deaminase activity.

Characterization of PGPR for plant growth promoting traits: Biochemical characterizations of selected PGPR strains (SH-4, SH-9 and SH-17) confirmed the presence of plant growth promoting traits (IAA production, P solubilization, ACC-deaminase activity, siderophore and phosphatase production) in these isolates but with variable extents. This variable potential among rhizobacteria for the production of IAA, siderophore, ACC deaminase and phosphatase may reasoned to differences as specific level or stains because similar variations have already been reported (Hayat *et al.,* 2013; Habibi *et al.,* 2014; Zahid *et al.,* 2015). P solubilization potential of PGPR generally depends on the amount and types of different organic acids such as lactic, isovaleric, isobutyric, acetic, gluconic, citric, oxalic, tartaric, succinic, and α-ketogluconic acids (Khan *et al.,* 2009). In this study we observed remarkable differences in the pH of media inoculated with different strains. The decrease in pH might be due to production of organic acids that eventually solubilize the tricalcium phosphate. These results were found in line with some previous studies. For example, Zhang *et al.* (2011) recorded variable ACC deaminase activity (213-370 µM α-ketobutyrate mg^{-1} h^{-1}), IAA (6.3-13.1 µg mL^{-1}), siderophore production (low to high), P solubilization (51-127 mg P mL^{-1}) in 8 different bacterial strains. Moreover, the group found negative correlation between pH of medium and soluble P contents. Similarly, Abbasi *et al.* (2011) screened rhizobacterial isolates on the basis of their IAA production that ranged between 5.5-31 µg mL^{-1}.

Effect of HA and PGPR on growth and yield of canola under field conditions: As plant height, root dry matter, number of lateral roots, number of pods $plant^{-1}$, number of seeds pod^{-1}, seed yield, 1000 seed weight and oil contents were remarkably improved with the application of HA and PGPR inoculation under field conditions. The impact of HA on plant height and other growth parameters may reasoned to its hormone-like activity and role in plant metabolism (Nardi *et al.,* 2009; Berbara & García, 2014).. It is an established fact that PGRs accelerate the vital developmental processes of cell elongation, cell division which cause improvement in plant growth (Campanoni & Nick, 2005; Panoli *et al.,* 2015). Likewise, PGPRs have been reported to enhance plant growth by the production of PGRs such as IAA, gibberellins, and cytokinins (Bhattacharyya & Jha 2012). *In vitro* production of IAA under axenic conditions by the *Acinetobacter* sp. used as PGPR in this study further strengthens our results. Moreover, both HA and PGPR have been reported to indirectly promote plant growth parameters by enhancing nutrient availability and uptake especially N which has significant role in the promotion of vegetative growth (Pii *et al.,* 2015; Metay *et al.,* 2015).

The improvement in number of pod $plant^{-1}$ and number of seeds $pods^{-1}$ of canola may also be attributed to the hormone-like activity of humic acids and PGRs production by PGPR which play crucial role in reproductive attributes of plants. Our findings are supported by earlier studies, which have reported PGRs for boosting of flowering, fruit development and seed setting (Pattison *et al.,* 2014). Besides, improved P uptake might be another important reason for enhanced number of pods $plant^{-1}$ and number of seeds pod^{-1} as it play important role in reproductive attributes (flowering, fruit set and fruit development) of crop plants (Havlin *et al.,* 2005).

Additionally, improvement in seed yield, 1000 seed weight, and oil contents of canola by humic acids and PGPR might be due to their role in improving above mentioned yield attributes as well as to their role in plant physiology and metabolism. Different researchers have reported the effects of HA and PGPRs on various physiological and metabolic processes, nutrients uptake and translocation and accumulation of photosynthates.

For example, El-Nemr *et al.* (2012) conducted a field trial on cucumber in Egypt for two seasons and noted that the humic acid considerably increased the number of flowers and fruit per plant, fruit set, average fruit weight, length and fruit diameter, and production per plant. Similarly, Yildrim (2007) observed enhancement of growth and production parameters due to the accumulation of soluble solids and ascorbic acid content in tomato by humic acid application. Karakurt *et al.* (2009) registered enhancement of total soluble sugars, reducing sugars and chlorophyll b contents in pepper with parallel improvement of growth and production by humic acid application. The findings of our work regarding the improvement of growth and production parameters by PGPR are also similar to Verma *et al.* (2011), Shahzad *et al.* (2013) and Zahid *et al.* (2105), who have reported improved growth and production of various crops by PGPR.

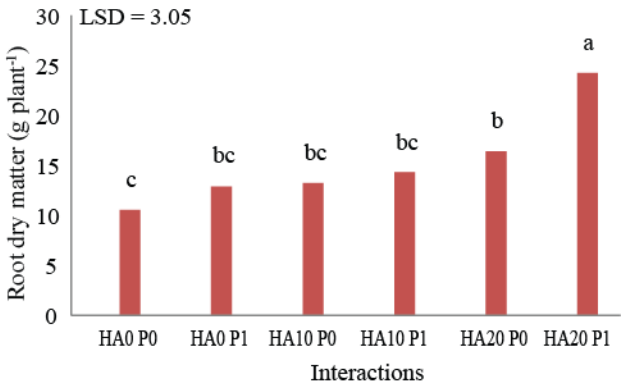

Fig. 3. Effect of HA and PGPR interaction on root dry matter plant^{-1}.

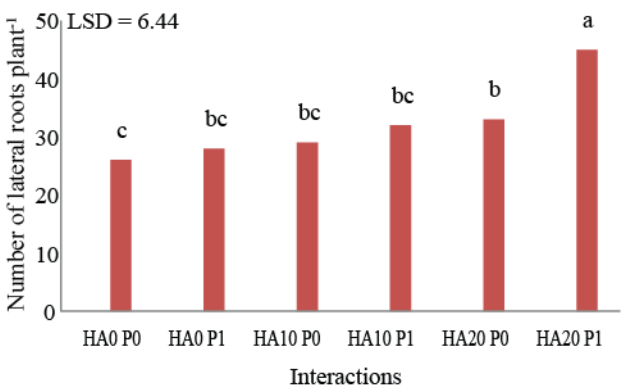

Fig. 4. Effect of HA and PGPR interaction on number of lateral roots plant^{-1}.

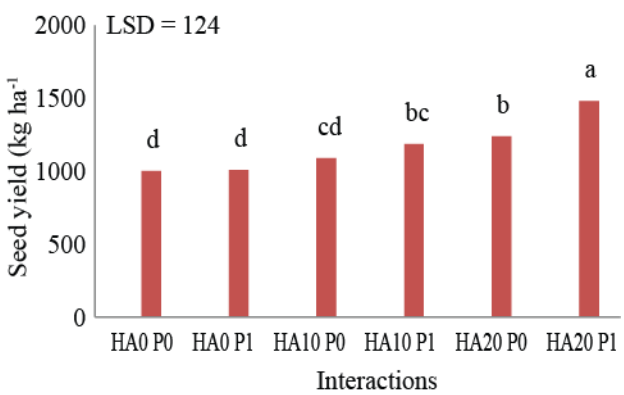

Fig. 5. Effect of HA and PGPR interaction on canola seed yield (kg ha^{1}).

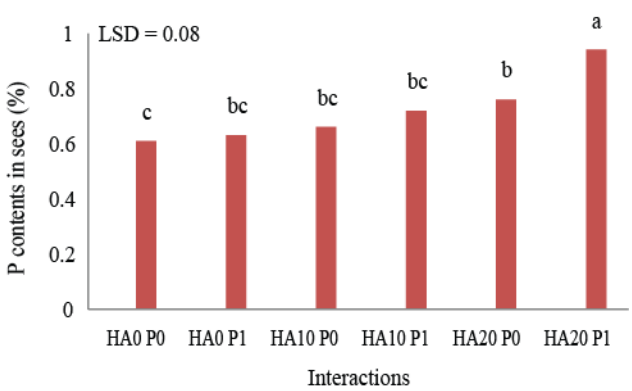

Fig. 6. Effect of HA and PGPR interaction on P contents (%) of canola seed.

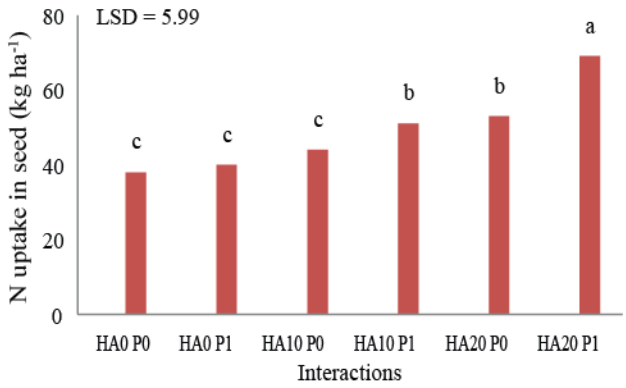

Fig. 7. Effect of HA and PGPR interaction on N uptake (kg ha^{1}) of canola seed.

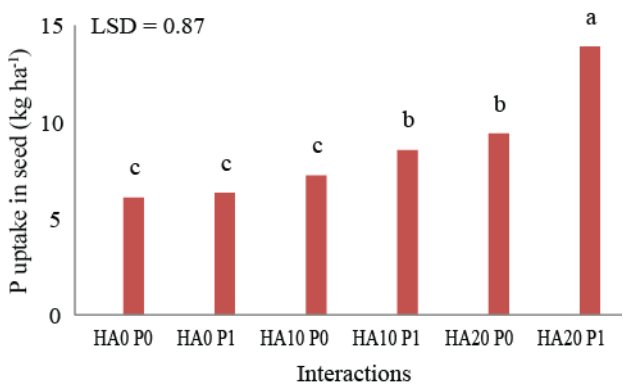

Fig. 8. Effect of HA and PGPR interaction on P uptake (kg ha^{-1}) of canola seed.

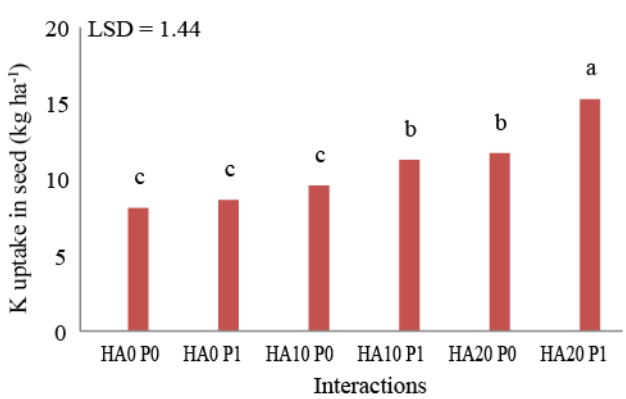

Fig. 9. Effect of HA and PGPR interaction on K uptake (kg ha^{-1}) of canola seed.

Fig. 10. Visual difference in root growth under 20 kg HA ha^{-1} x PGPR and 0 kg HA ha^{-1} x no PGPR interactions.

Effect of HA and PGPR on NPK concentration and uptake under field conditions: The enhancive effects of HA and PGPR on N, P and K contents and uptake in canola seed was observed compared to HA_0 and P_0. There are several possible mechanisms for improved plant nutrition like enhanced nutrient availability, increased nutrient uptake and better root growth. Humic acid enhances the nutrient availability through chelating, hence increasing their uptake (Nardi *et al*., 2009; Schiavon *et al.,* 2010). Similarly, humic acids also cause root elongation and promote lateral roots and root hairs (Canellas *et al.*, 2011; Jindo *et al.,* 2012). The better root growth is positively correlated to enhanced nutrient uptake as it helps the plants to take nutrient from larger volume of soil. The clear differences in root growth with and without HA and PGPR application were observed in this study (Fig. 10).

PGPR enhance nutrient availability through nutrient solubilization by releasing organic acids like lactic, isovaleric, isobutyric, acetic, gluconic, citric, oxalic, tartaric, succinic, and α-ketogluconic acids (Khan *et al.,* 2009). Production of enzymes (phytases and phosphatases) by PGPR also enhances nutrient availability by releasing the nutrients from organic matter (Jorquera *et al.,* 2008). The *In vitro* solubilization of P by PGPR observed in this study can justify their potential to improve nutrient availability by solubilization.

Similarly, enhanced root growth and architecture (root area, root length, root mass, number of lateral roots) by PGPR inoculation is also considered an important phenomena for improved nutrition (Calvo *et al.,* 2014). PGPR were documented to improve root growth in axenic and pot trials (Baig *et al.,* 2012; Shahzad *et al.,* 2013). Parallel improvement in nutrient uptake with better root growth by PGPR in different crops has been observed (Khan, 2005; Banerjee *et al.*, 2006; Yazdani & Pirdashti, 2011).

Furthermore, the PGPR (*Acinetobacter* sp.), used in this study has been found to possess P solubilization and ACC deaminase activities. Findings of Baig *et al.* (2012) also support our results who reported enhanced nutrient uptake by PGPR possessing ACC deaminase and P solubilization activities. Additionally, our results of improved nutrient contents and uptake by humic acid are supported by Morard *et al.* (2011) and El-Nemr *et al.* (2012). Similarly, enhancement of nutrient contents and uptake in different crops with PGPR application has also been documented by Shaharoona *et al.* (2008), Adesemoye *et al.* (2010) and Zahid *et al.* (2015). Furthermore, humic substances also positively influence the microbial community structure and function in the soil especially in the rhizosphere region (Varanini & Pinton, 2001). This might be the reason that we found interaction of HA_{20} x P_1 more efficient for nutrient uptake.

Conclusion

This research suggest that these could be considerable improvement of growth, yield and nutrition of canola with increment use of humic acid and PGPR inoculation. However, combined application of humic acid and PGPR was found much better than their individual effect. Especially, the interaction of 20 Kg HA ha^{-1} x PGPR effectively improve canola growth, yield and nutrition. Hence, HA and PGPR can be included in routine agronomic practices for maximizing the crop production on sustained bases in arid regions.

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