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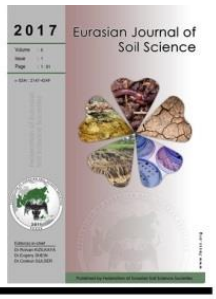

Comparative potential of *Rhizobium* species for the growth promotion of sunflower (*Helianthus annuus* L.)

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Abstract

Rhizobium besides its nodule formation characteristic with members of Fabaceae family has been recognized for its great root colonizing ability and growth hormone production potential. In addition to nitrogen fixation in legume plants, rhizobia considered as beneficial tools and act as plant growth promoting rhizobacteria (PGPR) with many non-legumes. Present study was elucidated to determine the comparative role of *Rhizbium* sp for growth promotion of sunflower. Rhizobia were isolated from five different legumes (mungbean, barseem, lentil, chickpea, and vegetable pea) and checked for their auxin production efficiency. Rhizobial isolates Cp-4 showed maximum auxin potential (5.37 µg mL^{-1}IAA equivalents).Results showed that inoculation of all rhizobial isolates caused significant increase in growth and physiological parameters of sunflower plants. While prominent results were found with inoculation of mungbean rhizobial isolate Mb-2 which increases the chlorophyll a, N, P, fresh and dry matter of sunflower significantly by 8.34, 4.9, 36, 31, and 34%, respectively in comparison to un-inoculated control plants. Hence, present study concluded that *Rhizobium* sp can be successfully used as PGPR in non-legumes after thorough investigations.

Keywords: Rhizobium sp, PGPR, auxin biosynthesis, growth, sunflower.

Introduction

Sunflower (*Helianthus annuus* L.) is an oil-seed crop grown in different ecologies of Pakistan. Pakistan is deficient in edible oil and fulfills 65% of its requirement by importing oils of worth 1.5 billion$. Sunflower oil is a rich source of linoleic acid (64%) that minimizes cholesterol level in the coronary arteries and has 40-45% high quality protein. Pakistan produced 13.4% sunflower oil domestically (Shah et al., 2005).

Microorganisms are considered as indicators of soil health. Plant growth promoting rhizobacteria (PGPR) influence the plant ontogeny by several means. It is well established that root exudates produced diverse organic substances important for rhizosphere microbes. Microbial colonization of plant roots influences plant ontogeny in a significant manner. Rhizobacteria stimulate the plant metabolic process and ultimately the plant growth. PGPR affected the plant growth by the production of plant-hormones, antibiotics, vitamins, suppression of plant pathogens and solubilization / mineralization of nutrients to more accessible forms for the plants. The root colonization of plants by PGPR greatly enhances the uptake of nutrients (Chen et al., 2005). Oilseed crops such as sunflower arehyper-accumulator and may be used to decontaminate the polluted soils and PGPR inoculation enhances their ability by number of ways (Kovar et al., 2016). *Rhizobium*, the most studied PGPR responsible for symbiotic nitrogen fixation in legumes may promote the

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growth of non-legumes. Literature confirmed the evidences of growth promotion of cereals, forages and fiber crops by *Rhizobium* sp (Hardoim et al., 2008; Qureshi et al., 2013).

Rhizobium species produced plant hormones such as indole-3-acetic acid (IAA), cytokinins, gibberellins and abscisic acid that regulated the endogenous levels of plant hormones, thus promote plant growth (Roy and Basu, 2004; Chi et al., 2005; Mehboob et al., 2008; Mehboob et al., 2009). Inoculation of *Rhizobium* sp improved the plant growth by solubilization of precipitated / fixed inorganic phosphates and mineralization of organic phosphates by producing organic acids and releasing phosphatase, enhance nutrient uptake and ultimately improve plant health (Biswas et al., 2000; Yanni et al., 2001; Alikhani et al., 2006; Hussain et al., 2009). *Rhizobium* sp promote growth by altering root architecture, producing siderophores and lowering ethylene level (Lupwayi et al., 2004; Madhaiyan et al., 2006; Mehboob et al., 2008; Qureshi et al., 2013) of non-legumes. Present study was designed to evaluate the comparative potential of *Rhizobium* species to promote the growth of sunflower.

Material and Methods

Isolations and purification of *Rhizobium* species

Rhizobium species of mungbean, berseem, chickpea, lentil and vegetable pea were isolated on yeast extract mannitol agar medium (YMA) (Vincent, 1970). The medium was autoclaved for 30 minutes at 121°C temperature and 15 psi pressure. The pouring of medium in petri plates was carried out in laminar air flow cabinets and exposed to UV for half an hour. The surface sterilization of collected nodules of different legumes (mungbean, berseem, chickpea, lentil and vegetable pea) was carried out as reported by Russell et al. (1982). The nodules were crushed separately to obtain suspension and streaked on YMA. The plates were labeled and incubated at 25°C in the incubator for 72 hours. The growth obtained was further purified on fresh plates having YMA containing 10 mL L^{-1} of 0.25% congo red. The purified isolates were preserved at 5± 1°C on slants for further screening.

Determination of auxin biosynthesis

Four purified isolates of each legume were assessed out for the auxin biosynthesis potential. Different isolates were labeled as mung bean (Mb-1, Mb-2, Mb-3, Mb-4), berseem (Br-1, Br-2, Br-3, Br-4), chickpea(Cp-1, Cp-2, Cp-3, Cp-4), Lentil (Lt-1, Lt-2, Lt-3, Lt-4), vegetable pea (Vp-1, Vp-2, Vp-3, Vp-4) during auxin estimation as IAA equivalents. Test tubes containing GPM were sterilized and inoculated with the respective isolates kept un-inoculated control, incubated at 28 ± 2 °C for one week and then centrifuged @1000 rpm for 10 minutes. The supernatants were analyzed colorimetrically using Salkowski reagent at 535 nm (Sarwar et al., 1992). Biochemical tests like congo red, bromothymol blue (BTB), oxidase test and gram reaction were carried out. Isolates having the highest auxin biosynthesis (Mb-2, Br-1, Cp-4, Lt-4 and VP-2) were selected for the pot experiment (Table 1).

Table 1.Auxin biosynthesis potential of isolates of *Rhizobium* species

Macrosymbiont	*Rhizobium species*	Isolates	IAA Equivalents (µg mL^{-1})
Mung bean	*Rhizobium phaseoli*	Mb-1	4.02
		Mb-2	5.11
		Mb-3	4.91
		Mb-4	4.64
Bserseem	*Rhizobium trifolii*	Br-1	4.03
		Br-2	3.33
		Br-3	3.30
		Br-4	3.81
Chickpea	*M. ciceri*	Cp-1	4.40
		Cp-2	4.23
		Cp-3	3.37
		Cp-4	5.37
Lentil	*R. Leguminosarum*	Lt-1	3.10
		Lt-2	3.83
		Lt-3	3.95
		Lt-4	4.20
Vegetable pea	*R. Leguminosarum bv.viciae*	Vp-1	3.81
		Vp-2	4.03
		Vp-3	3.64
		Vp-4	3.30

Preparation of inocula

The broth medium of YEM was prepared and sterilized. The sterilized medium was inoculated by respective isolates and placed on orbital shaker and after gaining optimum growth incubated at 28 ± 2 °C. The well decomposed leaf mold was sterilized and inoculated with broth cultures of *Rhizobium* species. Bacterial inocula were prepared by adding 20% sugar solution and incubated at 28 ± 2°C to enhance the respective bacterial population up to 10^8 CFU $g^{-1}$and applied to seeds as seed coating.

Pot experiment

Pot study was conducted to evaluate the comparative potential of *Rhizobium* species to promote the growth of sunflower using the completely randomized design (CRD). The pre-sowing soil was medium textured having pH 7.88, EC 1.40dS m^{-1}, soil N 0.035 % and available P 7.42 mg kg^{-1} at Soil Bacteriology Section, AARI, Faisalabad with three replication. Uniform fertilizer @ 50 kg N ha^{-1} and 75 kg P ha^{-1} was applied. After one month of sowing, the rhizosphere soil and leaves were collected to assess the IAA equivalents in the rhizosphere (Sarwar et al., 1992) and chlorophyll content as reported by (Arnon, 1949). Data regarding biomass, dry matter, N and P content in plant and post-harvest soil N and available P were determined. Chlorophyll content (a and b) of leaf was determined as reported by Arnon (1949). Soil samples were analyzed for extractable P (Olsen and Sommers, 1982) and soil N by (Bremner and Mulvaney, 1982). Data were subjected to statistical analysis following completely randomized design (CRD) (Steel et al., 1997) and differences among the treatments means were compared by the Duncan's multiple range test (Duncan, 1955).

Results

Lab study was conducted to test the biosynthesis of auxins as IAA equivalents by different rhizobial isolates and isolates i.e. microsymbionts of different legumes showed promising results were selected to assess their sunflower growth promotion potential. Results (Table 1) shown that rhizobial isolates Mb-2, Br-1, Cp-4, Lt-4 and Vp-2 produced auxins i.e. 5.11, 4.03, 5.37, 4.20 and 4.03 µg mL^{-1}as IAA equivalents, respectively. However, rhizobium sp of chickpea produced the maximum IAA production efficacy i.e. 5.37 µg mL^{-1}IAA equivalents.

Results regarding fresh and dry matter yield were presented in Figure 1. Bacterial inoculation of IAA-producing different rhizobial isolates showed positive influence on sunflower growth significantly and enhanced fresh and dry biomass in comparison un-inoculated control. The highest increase in fresh and dry biomass was obtained by Mb-2 inoculation that increased these parameters significantly by 31 and 34% followed by Br-1 i.e. 25 and 22%, respectively than control. The rhizobial isolates Cp-4, Lt-4 and Vp-2 increased these parameters by 23 and 17%, 17 and 7%, 8 and 4%, respectively compared to control.

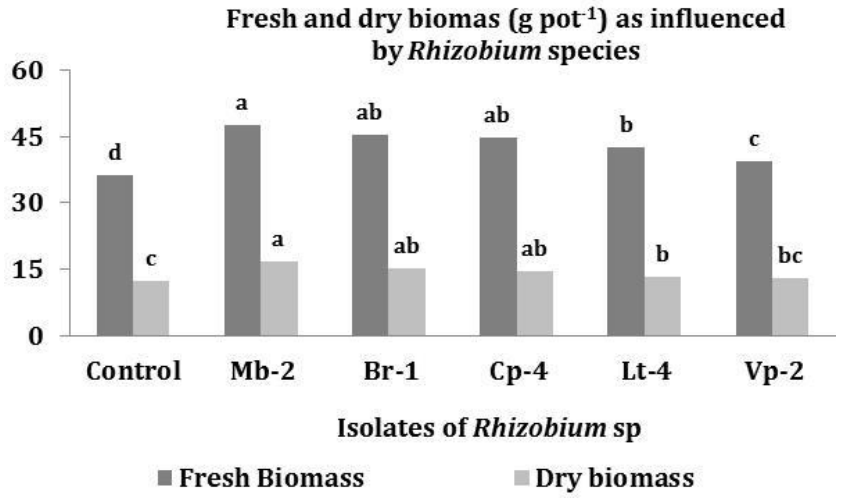

Figure 1. Fresh and dry matter biomass of sunflower as influenced by different treatments.

The data regarding chlorophyll contents and IAA equivalents in the rhizosphere soil presented in Figure 2 and 3. The extent of improvement in chlorophyll contents and IAA equivalents showed variable response with inoculation of different species. The maximum response was observed when inoculation was done with Mb-2 which increased chlorophyll a contents by 1.4 mg g^{-1} (8.34%) significantly while minimum improvement (1.33 mg g^{-1}) in chlorophyll a was noted with Vp-2 than control. In case of chlorophyll b, again Mb-2 gave better response than the rest of isolates under study i.e. 0.643 mg g^{-1} (5%) than control. Rhizobial isolate Mb-2 also gave best performance in case of IAA equivalents and gave higher content of IAA equivalents in the rhizosphere(4.56 µg g^{-1})while the rest of isolates (Br-1, Cp-4, Lt-4 and Vp-2)are statistically non-significant to each other but higher than control. i.e. (4.17, 4.25, 3.98 and 3.82 µg g^{-1}), respectively as compared to control i.e. 3.74.

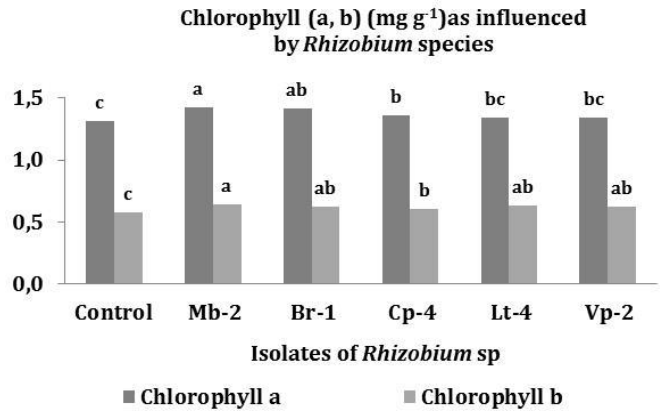

Figure 2. Chlorophyll (a & b) content as influenced by different rhizobium isolates

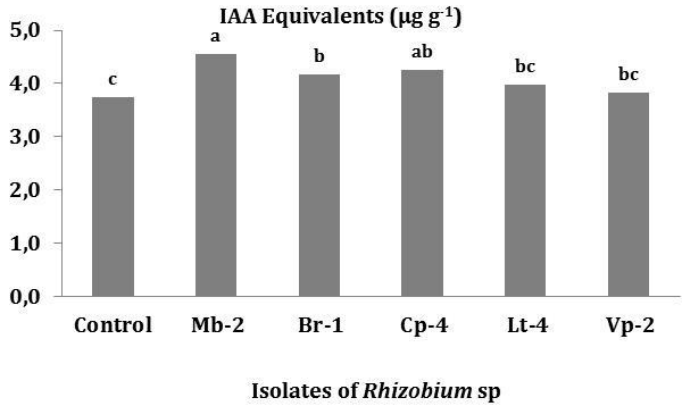

Figure 3. IAA equivalents in the rhizosphere (µg g^{-1}) as influenced by different rhizobium isolates

Data regarding plant N and P content was presented in Figure 4 and 5. Rhizobium sp isolates increased the N and P content significantly and Mb-2 gave much significant increase of 4.9 and 36% in N and P of sunflower plants, respectively than un-inoculated control.Isolate Vp-2 remained least effective. Other rhizobial isolates including Br-1, Cp-4 and Lt-4 enhanced the N and P content by 3 and 27%, 4 and 27, 2.4 and 18%, respectivelythan control. However, response of rhizobial isolates Br-1, Lt-4 and Vp-2 remain at par with each other regarding plant N and P content.

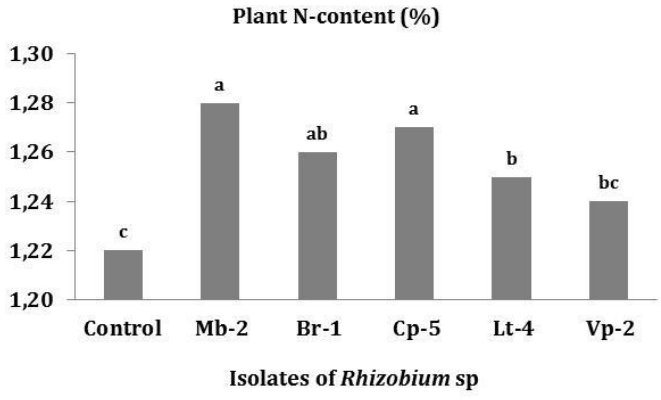

Figure 4. Effect of different treatments on plant N content

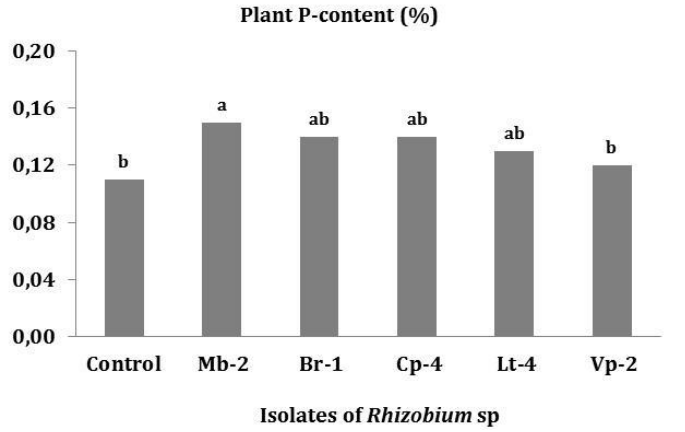

Figure 5. Effect of different treatments on plant P content

Results regarding soil N and available P contents in soil are presented in Figure 6 and 7. Results revealed that inoculation of IAA-producing rhizobial isolates exerts positive influence on soil nutritional status and enhanced soil N and available P content. Likewise, the isolate Mb-2 significantly increased soil N and available P by 11.76 and 13.26%, respectively than un-treated control. However, the effect of Mb-2 on soil N and P levels was non-significant compared to Br-1 and Cp-4.

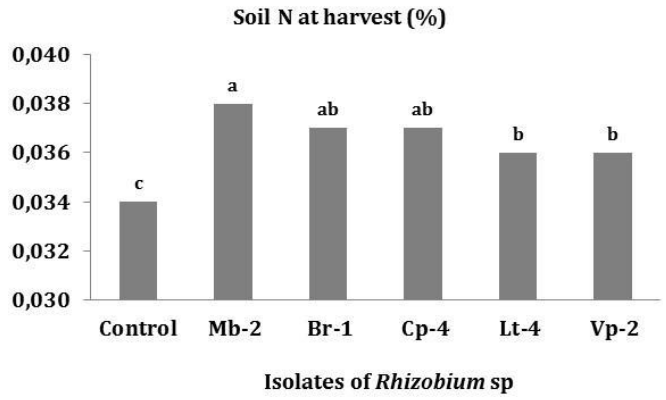

Figure 6. Effect of different isolates on post harvest soil N

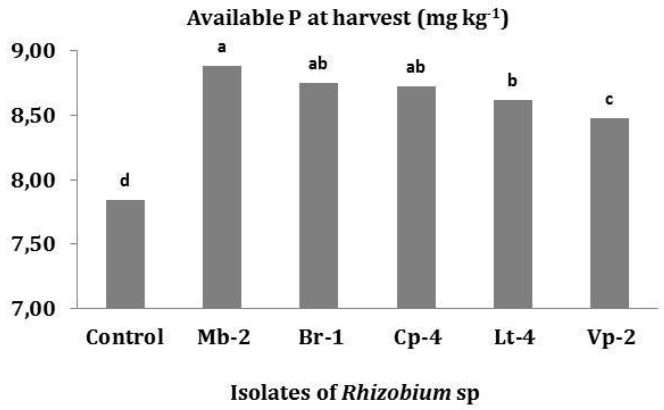

Figure 7. Effect of different isolates on post harvest soil available P content

Discussion

Different *Rhizobium* sp isolated from different legumes are reported to make an association with non-legumes and act as PGPR (Hussainet al., 2009; Mia and Shamsuddin, 2010; Qureshi et al., 2013;Naveed et al., 2015a; Adnan et al., 2016). *Rhizobium* species as PGPR possess various mechanisms which are responsible for stimulating plant growth such as production of phytohoromone, siderophores, cyanide, killing harmful pathogens by lytic enzymes, antibiotics, enhancing micro and macro-nutrients mobilization like phosphate solubalization, quorum-sensing signal interference, organic compounds, inducing systemic resistance, nitrogen fixation, biofilm formation, releasing ACC deaminase and symbiotic relation between plant and microbes (Alamiet al., 2000; Compant et al. 2005; Bhattacharyya and Jha, 2012; Adnan et al., 2016; Jimenez-Gomez et al., 2016).

Present study was conducted to test rhizobium species from five different legumes and for growth promotion of sunflower. Isolates of different species were isolated and checked for auxin biosynthesis potential. The tested isolates produced auxin as IAA equivalents with variable amount on respective media. Our results corroborated the results of Adnan et al. (2016) who isolated rhizobia from nodules of five different summer legumes and found that 47% of isolated rhizobial species were capable of producing IAA.

The isolates having maximum potential of auxin biosynthesis produced fresh and dry biomass of sunflower plants significantly higher than control. Increase in fresh and dry biomass owed to the production of phytohoromones, siderophores, hydrogen cyanide (HCN), solubilization of phosphates (Sessitsch et al., 2002; Hussain et al., 2009;Sachdev et al., 2009; Mia and Shamsuddin, 2010; Naveed et al., 2015a). Furthermore, biocontrol activity of *Rhizobium* sp. i.e.*B. japonicum*, *R. leguminosarum* and *S. meliloti* against *Macrophomina phaseolina*, *Fusarium* sp and *R. solani*in crop plants (Compant et al., 2005; Mehboob et al., 2008; Mia and Shamsuddin, 2010). Our result are in agreement with Alami et al. (2000) that demonstrated significant increase in root and shoot dry matter (50 and 70%, respectively) of sunflower plants in both drought and irrigated conditions when seeds were inoculated with exopolysaccharide (EPS) producing *Rhizobium* sp.

Inoculation of different rhizobial isolates increased the chlorophyll contents of sunflower because due to the microbial production of siderophores andsiderophores bind with Fe and facilitates its availability to plants (Carson et al., 2000; Barry and Challis, 2009; Dimkpa et al., 2009; Naveed et al., 2015a). The bacterial production of siderophores enhanced chlorophyll content and growth of plants due to the selective iron uptake from the solutions of trace elements and also production of siderophores inhibited the free radical formation and substantiated for the oxidative stress and prevented the uptake of heavy metals (Barry and Challis, 2009; Dimkpa et al., 2009; Naveed et al., 2015a). The substantial increase in chlorophyll contents of wheat plants treated with PGPR strains *B. phytofirmans* PsJN was reported by Naveed et al. (2014). A significant increase in plant IAA level of plants might be due to natural IAA-producing capability of selected rhizobial isolates (Khalid et al., 2006: Perrine-Walker et al., 2007; Naveed et al., 2015b). Chi et al. (2005) reported similar findings in which rice seedlings were inoculated with *A.caulinodans* ORS571 and *S. meliloti* 1021 and found substantial increase in levels of gibberellins (GA3) and auxins (IAA) in leaves and leaf sheath of rice plants.

Application of selected plant growth promoters enhanced NP contents of sunflower plants as well as in soil that might be due to associative relation of rhizobia with non-legumes. Microbial inoculation enhanced the nutrient uptake by altering the root system architecture, colonizing roots, producing hormones / siderophores, mobilizing nutrients and also enhanced root exudation (Chalk, 2016; Paungfoo-Lonhienne et al., 2014). Except biological nitrogen fixation, rhizobia also enhance nitrogen uptake in non-legumes such as rice (Biswas et al., 2000). Different isolates belongs to the group of PGPR viz. *Rhizobium*, *Enterobactor*, *Pseudomonas* and *Bacillus* are recognized as P-solubilizer (Shahid et al., 2012). Microbes produced organic acids that responsible for slight decrease in rhizosphere pH and theses organic acids acted like chelates and enhances nutrient uptake. Furthermore, organic acids possess carboxyl and hydroxyl groups which chelate or detach cations (Ca, Mg, Fe, Al) from soil phosphate eventually making it available for plants (Mullen 2005; Shahid et al., 2015).

Some *Rhizobium* sp like *R. leguminosarum* and *S. meliloti* are reported to release riboflavin i.e. vitamin riboflavin converted into lumichromeeither photochemically or enzymatically and motivated root respiration (Dakora et al., 2002; Yang et al.,2009; Qureshi et al., 2013; Naveedet al., 2015a) ultimately facilitating nutrient uptake. Additionally, rhizobia might enhance nutrients availability through release of various chemical compounds like exopolysaccharide, phytohoromones, lipo-chitooligosaccharides (LCO's), siderophores and lumichromes (Mehboob et al., 2008; Mehboob et al., 2009; Naveed et al., 2015a). Biswas et al. (2000) depicted a significant increase (up to 28%) in nutrients (N, P and K) uptake when they applied different rhizobial strains to rice seeds / seedlingscompared to un-inoculated control. Our results are also corroborated with the findings of Shahid et al. (2015) who applied two P-solubilizing bacterial strains (*Alcaligenes faecalis* Ss-2and *Bacillus* sp Ps-5) to sunflower seeds and reported a significant increase in P contents of sunflower plants in comparison to control. Alami et al. (2000) also observed that application of EPS producing rhizobial strains YAS34 increased water and nitrogen nutrition of sunflower under drought. Furthermore, Matiru and Dakora (2004) reported significant increase in P uptake and plant growth was observed through application of different rhizobia to sorghum plants.

Present study concluded that *Rhizobium* species can be successfully used as PGPR after thorough screening and by determining its growth hormone production potential. Present study also presented new horizons about variable response of different crop specific rhizobium species. Furthermore, study concluded that Rhizobium species having growth hormone producing potential might enhance plant growth and development, nutrient uptake and mobilizer.

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