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Research Article

ISOLATION AND CHARACTERIZATION OF INDOLE ACETIC ACID PRODUCING PLANT GROWTH PROMOTING RHIZOBACTERIA FROM RHIZOSPHERIC SOIL OF *WITHANIA SOMNIFERA*

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ABSTRACT

Indole acetic acid (IAA) production is a major property of rhizosphere bacteria that plays a central role in plant growth and development as a regulator of cell division, cell differentiation, cell expansion, lateral root formation, flowering, and tropic responses. The present study deals with isolation, functional characterization and identification of IAA producing rhizobacteria from the rhizosphere. The present study deals with the emerging need to protect medicinal plants that represent our natural resources. A total ten bacterial isolates were recovered from rhizospheric soil associated with *Withania somnifera*, recognized as *bacillus spp.* by morphotpic and biochemical characterization and tested for indole acetic acid production. Out of ten rhizobacterial isolates, six were selected as efficient Producers of IAA. The amount of indole acetic acid produced was detected in the presence of different concentration of tryptophan, which lead to the elevation in indole acetic acid production as compared in the absence of tryptophan. The IAA production was further confirmed by thin layer chromatography (TLC). The impact of PGPR was evaluated on *Withania somnifera* seeds that showed significant elevation in germination %, root and shoot length as compared to the untreated seeds. Out of the chosen PGPR, WSNb3, WSNb5 and WSNb6 were showed to be the best to produce IAA. Subsequently, effect on plant growth was tested by towel paper assay. In conclusion the study suggests the IAA producing bacteria may be efficient biofertilizer inoculants to promote plant growth and protecting the medicinal plants for the future generation.

Keywords: IAA, phytohormone, rhizobacteria, biofertilizers, TLC.

INTRODUCTION

Medicinal plants represent one of the most valuable resources of ancient Indian culture. The demand for the medicinal products is increasing today, because most of the population is dependent on the herbal, Ayurvedic medicines made from the medicinal plants. This is due to plant products being natural, non toxic and efficient. Therefore, there is a sudden need to protect out natural resources to meet the need for various medicinal purposes and for the future generation. The most efficient technique can be use of natural soil microorganisms present in the rhizosphere of root and provide beneficial effect for the plant growth. Rhizospheric microbes produce plant hormones and communication molecules that all encourage plant growth. Microbial population in rhizosphere may benefit the plant in a variety of ways including increased recycling and solubilization of nutrients, amino acids, synthesis of vitamins, auxins, cytokinins and gibberellins which enhance plant growth and antagonism with potential plant pathogens through competition and development of amensal relationships based on production of various antibiotics. The main reason of microbial specificity towards the various medicinal plants could be due to exchange of plant metabolites[1]. The action and interaction of some growth regulators like auxins regulate most of the activities and growth in plants. Naturally occurring substances with indole nucleus possessing growth-promoting activity are referred to as auxins. Chemically it is Indole acetic acid and 80 % of the bacteria isolated from plant rhizosphere are to produce IAA[2]. Rhizobacteria identified and tested to produce IAA from producing freshwater wetland rhizosphere associated with *Juncus effusus*[3]. IAA is phytohormone which is very commonly produced by PGPR[4] and production of this hormone and implicated it in the growth promotion of various plant by PGPR[5]. However, the effect of IAA production on plants health depends on the plant sensitivity to IAA and the amount of IAA produced from plant associated rhizobacteria and induction of other phytohormones. IAA produced from *P. putida* played a major role in the development of host plant root system[6]. Indole-3-acetic acid (IAA) is known to regulate many aspects of plant development some of them include the differentiation of vascular tissues, lateral root initiation, elongation growth, apical dominance, fruit setting and ripening[7]. IAA production by bacterial isolates has been recognized as the most important factor in direct growth promotion of root and shoots length of plants. Rhizosphere microorganisms associated with various plants synthesize and release auxin as secondary metabolites because of the rich supplies of substrates exuded from the roots compared with non rhizospheric soils[8]. It has ben observed that phytohormone producing root colonizing bacteria when bound to the seed coat of a developing seedling may act as a mechanism for plant growth stimulation[9]. In response to root exudates, the root associated microorganisms including symbiotic species within the genera *Rhizobium* synthesize indole acetic acid[10]. Indole acetic acid (IAA) is one of the most physiologically active auxins. IAA is a most common

product of L-tryptophan metabolism produced by various microorganisms including Plant Growth-Promoting Rhizobacteria (PGPR)[11]. Rhizosphere strains consuming of root exudates including available Trp and it is converted to IAA, whereas plant and rhizobacteria may regulate the IAA biosynthesis in the rhizosphere[12]. It has been reported that with an increase in concentration of tryptophan from 1 to 5 mg/ml, the production of IAA by fluorescent *Pseudomonas* isolates also increased[13]. IAA is phytohormone which is known to be involved in root initiation, cell division and cell enlargement[14]. This phytohormone is very commonly produced by PGPR. Bacteria that colonize the rhizosphere and plant roots, and stimulate plant growth by any mechanism are referred to as PGPR[15]. PGPR can exhibit a variety of characteristics responsible for influencing plant growth. Bacterial production of IAA suggests that the pathways involved in IAA production may play an major role in defining the impact of the bacterium on the plant. It has been observed that most of the pathogenic strains of bacteria synthesized IAA via the indoleacetamide pathway while plants utilize the indolepyruvic acid pathway[16]. This helps the bacteria to evade plant regulatory signals and so the IAA induces uncontrolled growth in plant tissues. In contrast the useful bacteria such as PGPR synthesize IAA via the indole pyruvic acid pathway and the IAA secreted is thought to be strictly regulated by the plant regulatory signals. Differences in the production of IAA among bacterial strains can be attributed to the various biosynthetic pathways, location of the genes involved, regulatory sequences, and the presence of enzymes to convert active free IAA into conjugated forms. It is also dependent on environmental conditions. The objectives of this experimental research is to study the IAA hormone production of PGPR under laboratory condition and to determine effect of IAA hormone on plant growth promotion of *Withania somnifera* because IAA production by isolates has been recognized as the most important factor in direct growth promotion of plants. It has been reported that colorimetric method as a simplest method and has long been employed for the detection of indole-3-acetic acid produced by plants and microorganisms[17].

MATERIAL AND METHODS

Soil sampling

Rhizospheric soil samples were collected from *Withania somnifera* (ashwagandha) growing in fields of Uttarakhand University of horticulture and forestry (UUHF), Bharsar, Pauri Garhwal, India. Intact root system was dug out and the rhizospheric soil samples were carefully taken in plastic bags and stored at 4°C.

Isolation of rhizobacteria

Rhizosphere soil samples were collected from Ashwagandha growing fields in Bharsar, Pauri Garhwal, India. Bacteria were isolated using dilution method with nutrient agar medium (Peptone 5, Sodium chloride 5, Beef extract 3 and Agar 15 g/ltr. Final pH (at 28°C). Rhizosphere soil samples (10 g) was suspended in 90 mL of 0.85 % normal saline (pH 7.0) and shaken vigorously at 150 rpm at 37°C for 1 h. The resulting sample was serially diluted (100 µL) to 900 µL of 0.85 % normal saline in each Eppendorf tube and appropriate dilution (10^{-5}) of this suspension (0.1 mL or 100 µL) was spread plated in triplicate on NB medium. Cultures were incubated at 37°C ± 2 for 2 d. For experimental use, isolates were transferred when needed to nutrient agar medium that was stored at 4°C. Each colony was assayed further for morphological and physiological characteristics including Gram reaction, endospore and catalase enzyme activity. Bacterial isolates were identified by morphological and biochemical characteristics based on Bergeys' Manual of Systematic Bacteriology[18].

Morphological Characterization

Recovered bacterial isolates were phenotypically (morphotypic and functional) characterized. A total 10 of isolates were thus randomly selected morphologically from Ashwagandha rhizosphere. Colony morphology of isolates was studied under a microscope. This included shape, edge, elevation, surface and pigmentation. These characters are observed after the incubation period of the bacterial cultures on the solid media. In liquid cultures, we can observe the pellicle and sediment formation. Biochemical characteristics include enzyme production. Cellular morphology was based upon cell shape and Gram staining and endospore staining. Bacteria identification was carried out on the isolates by comparing the results obtained with that of Bergey's manual of determinative systematic bacteriology (1986).

Qualitative Screening for *in vitro* Indole acetic acid (IAA) production

A loopful of bacteria was inoculated and incubated into pre-sterilized Peptone broth containing 1 % of trypton for 48 h at 37^0C. After 48 hours add 1 ml of kovac's reagent to all tubes including control and shake after 15 minutes. The appearance of red ring at the top is the clear indication of indole acetic acid production.

Quantitative Estimation of IAA Production from Rhizobacteria

Production of IAA was performed using the Quantitative analysis [19] at different concentrations of tryptophan (0, 50, 100, 200, 500 µg/ml). Bacterial cultures were grown for 48 h in nutrient broth. Fully grown cultures were centrifuged at 4500 rpm for 15 minutes at 4^0C. The supernatant (1 ml) was mixed with 4 ml of Salkowaski reagent (50 ml, 35 % of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Samples were left at 28^0C for 25 minutes. Development of pink color indicates production of IAA. Optical density was taken at 535 nm with a spectrophotometer. Concentration of IAA produced by bacterial cultures was measured with the help of standard graph of IAA.

Confirmation of IAA by using TLC

A total six rhizobacterial cultures were inoculated in nutrient broth amended with 5 mM tryptophan. 1 % bacterial inoculum of O.D. 600 was used for inoculation. The inoculated nutrient broth was incubated at 28^0C for 24 h. After 24 h of incubation, nutrient broth was centrifuged at 7000 rpm for 10 minutes. 1:2 aliquots of liquid portion of centrifuged sample were extracted with

ethyl acetate. The organic phase was concentrated to dryness and then diluted with 0.5 ml methanol. This sample along with the standard IAA was applied on silica gel plate and TLC was run by using a solvent system such as propanol: water (8:2) proportion and developed by using Salkowaski reagent. Red colour spots were developed. R_f value of the standard and IAA produced by the selected isolates was calculated.

Effect of IAA producing rhizobacteria on plant growth by Roll Towel Method

To study the effect of IAA producing rhizospheric isolates on plant growth, roll Towel assay was performed. The experiment was conducted to assess the influence of six efficient rhizobacterial isolates for their plant growth promotion ability by the standard roll towel method[20,21] in growth chamber. Bacteria were grown in nutrient broth medium on a shaker (150 rpm) for 2 days and centrifuged at 10,000 rpm for 5 minutes. The pellet was mixed with sterile carboxy methyl cellulose (CMC) (HiMedia) suspension (1 %). *Withania somnifera* seeds were surface sterilized with 0.1 % mercuric chloride for 5 minutes, rinsed with sterilized distilled water (SDW) and soaked in bacterial suspension (3×10^8 cfu ml^{-1}) using 1 % carboxymethyl cellulose (CMC) for 24 h and sterile blank nutrient broth served as control. Then the seeds were blot dried, placed in wet blotters and incubated in plant growth chamber maintained at 25 ± 2^0C and 95 ± 3 % RH. Each rhizobacterial treatment was replicated four times with 40 seed per each. The percentage of germination was recorded after fifth day. Ten seedlings were taken at random from each replication and length of root and shoot measured separately at 21 days. Plant growth promotion of selected isolates was assessed on *Withania somnifera* seedlings using Vigour Index (VI)[22].

Vigour index (VI) = Seedling length (Mean root length x mean shoot length) x Germination %

RESULTS AND DISCUSSION

Isolation and Biochemical Characterization of Rhizobacteria

A total of ten rhizobacteria were isolated from rhizospheric soils in Pauri, Garhwal region, India and they were identified on the basis of morphological and biochemical characteristics among which six were selected based on IAA production ability. They were recognized as *bacillus* species. Bergey's manual of determinative of bacteriology was used as a reference to identify the isolates. The isolates were coded as WSNb1, WSNb2, WSNb3, WSNb4, WSNb5 and WSNb6 (from *Withania somnifera* rhizosphere in nutrient agar)

Table 1: Morphological and Biochemical characterization of IAA producing rhizobacterial isolates recovered from *Withania somnifera*

characteristics	Isolates from rhizosphere					
	WSNb1	WSNb2	WSNb3	WSNb4	WSNb5	WSNb6
Gram staining	Positive	Positive	Positive	Positive	Positive	Positive
Endospore	Positive	Positive	Positive	Positive	Positive	Positive
Shape	Rod	Rod	Rod	Rod	Rod	Rod
Form	Irregular	Round	Irregular	Irregular	Round	irregular
Colour	Yellow	Yellow	Yellow	Yellow	Cloudy	Cloudy
Catalase	Positive	Positive	Positive	Positive	Positive	Positive
Oxidase	Negative	Negative	Negative	Negative	Negative	Negative
Gelatinase	Positive	Positive	Positive	Positive	Positive	Positive
Amylase	Positive	Positive	Positive	Positive	Positive	Positive
Citrate	Positive	Positive	Positive	Positive	Positive	Positive

Screening for Qualitative Indole Acetic Acid (IAA) production

All the isolated organisms identified as *bacillus* spp. were screened for their ability to produce IAA. Among all isolated bacteria 6 of them showed red colour reaction with kovac's reagent indicating their ability to produce IAA as shown in (Figure 1). The production of phytohormone IAA is the indication of beneficial effect by the rhizobacteria on plant growth stimulation. So these are better termed as plant growth promoting rhizobacteria (PGPR).

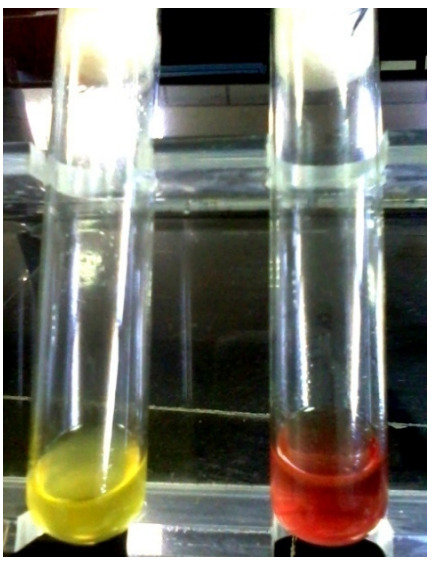

Figure 1: Indole acetic acid production by PGPR isolates

Quantitative estimation of IAA produced

A total of 6 bacterial isolates were used for the quantitative estimation of IAA in the presence of different concentrations of tryptophan (0, 50, 100, 200 and 500 µg/ml). The amount of IAA produced at different concentration of tryptophan was estimated by comparing with the standard calibration curve. The amount of IAA produced by all the 6 isolates was estimated as shown in Table 2

Preparation of standard graph of IAA

The standard IAA calibration curve was set up by determining the prepared different concentrations of authentic IAA at 535 nm with UV spectrophotometer as shown in (Figure 2) from this result; the best IAA producers were selected for further study.

Effect of tryptophan concentration

L-Tryptophan is generally considered as an IAA precursor; because of its addition to IAA producing bacterial culture enhances IAA biosynthesis[23]. All 6 isolates preferred Tryptophan for IAA production. In the absence of L-tryptophan, all the selected PGPR isolates produced very low amount of IAA ranged from 1 to 3.5 µg/ml as represented in the (Figure 3). In the presence of L-tryptophan the concentration of IAA produced by the rhizobacterial isolates ranged from 5 to 11 µg/ml as shown in (Figure 4). In present study it was observed that as the concentration of tryptophan in the medium increases, the amount of IAA produced increased. The order of IAA produced in the presence of tryptophan is WSNb3 >WSN b6> WSNb5 > WSNb4 > WSNb2> WSNb1. All the isolates showed less IAA production in absence of tryptophan, which concludes the requirement of tryptophan as a precursor for the synthesis of IAA. The production of IAA by tryptophan is done by two pathways viz through indole-3-pyruvic acid and indole-3-acetamide.

IAA confirmation by thin layer chromatography (TLC)

Purified IAA sample was compared with standard IAA on TLC chromatograms. TLC of ethyl acetate extract showed pink colour spot at the R_f corresponding to the standard IAA (0.9) as shown in (Figure 5). It confirmed IAA producing potential of rhizospheric isolates.

Evaluation of effective PGPR strains on ashwagandha seeds by Roll towel method

The growth promoting activity of 6 isolates of PGPR was tested for seed germination and Seedling vigour by using ashwagandha seeds in roll towel methods. In roll towel method, the PGPR strains WSNb3, WSNb5 and WSNb6 produced higher shoot and root length of ashwagandha seedlings (Figure 6) and showed more than 90per cent seed germination (Figure 7) and with enhanced vigour index after 21 days. The maximum vigour index of 1035.5 was recorded in WSNb3 treated seedlings followed by 936 and 893 vigour index in WSNb5 and WSNb6 treated seedlings respectively (Figure 8) Least germination (80 %), less shoot and root length of seedlings with less vigour index of 424 was observed in untreated control (Table 2). The promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the rhizosphere and other PGP activities[24].

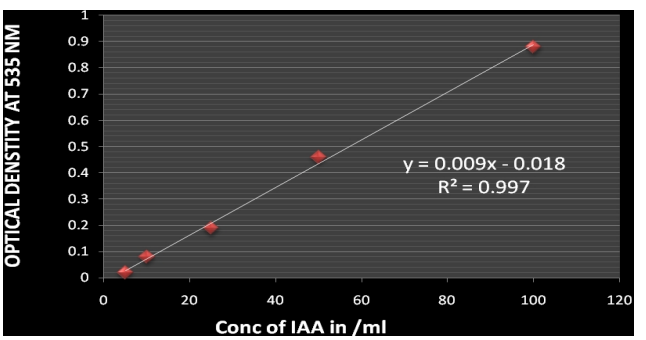

Figure 2: Standard graph of IAA

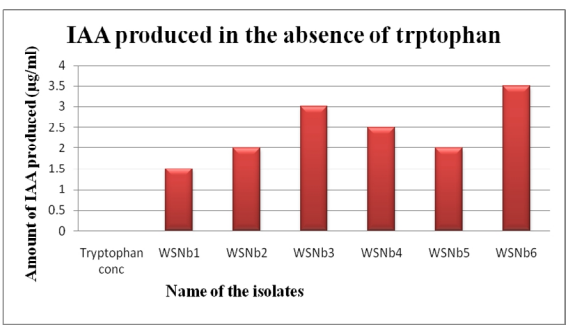

Figure 3: Effect on IAA production (µg/ml) by isolates in the absence of tryptophan

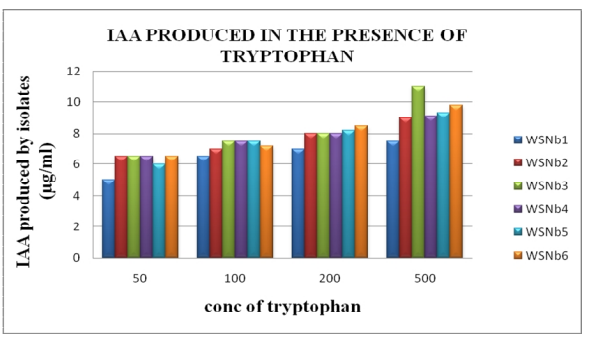

Figure 4: Effect on IAA production((µg/ml) by isolates in the presence of tryptophan

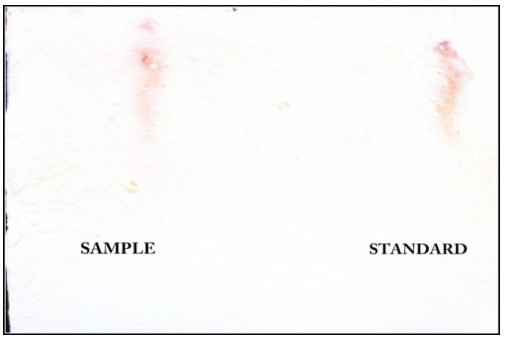

Figure 5: Thin layer chromatogram of IAA detected from PGPR isolates by Salkowaski's reagent compared with standard

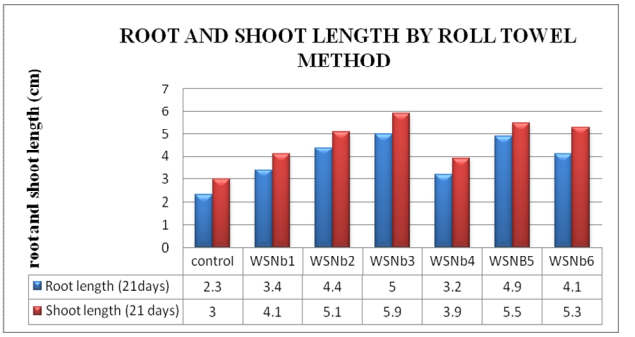

Figure 6: Effect of rhizobacterial isolates on root length and shoot length by roll towel method

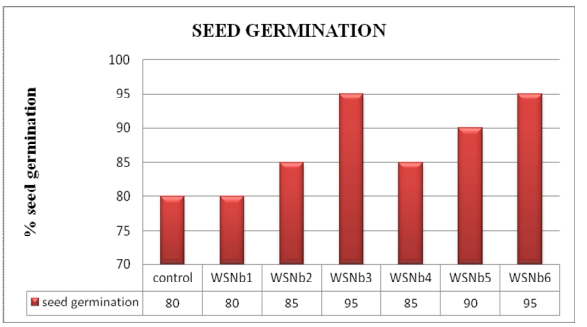

Figure 7: Effect of rhizobacterial isolates on seed germination

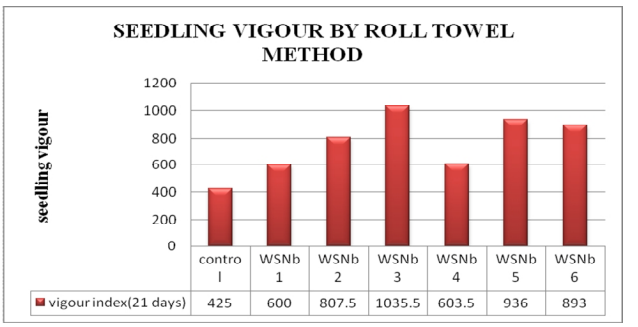

Figure 8: Effect of rhizobacterial isolates on seedling vigour by roll towel method

DISCUSSION

IAA, a member of the group of phytohormones, is generally considered as the most important native auxin. All ten rhizospheric isolates were recognized as bacillus and also are positive for IAA production but among those six isolates WSNb1, WSNb2, WSNb3, WSNb4, WSNb5 and WSNb6 were selected as potential IAA producers. *Bacillus* species were the dominant isolated bacteria in the rhizosphere of *Withania somnifera* as a medicinal plant. In the similar study, various bacteria isolated from rhizosphere of 50 medicinal plants which among the isolated bacteria the dominant species was *Bacillus* followed by *Enterobacter, Corynebacterium, Pseudomonas, Micrococcus* and *Serratia*[25]. The use of the technique for the detection of IAA using the Salkowski reagent is an important option for qualitative and semi-qualitative determination that assure the presence of the hormone in the supernatant of bacterial cultures or liquid formulations of bioinoculants. The amount of IAA produced by the rhizobacterial isolates were within the detection limits of Salkowski reagent[26]. The reagent gives reaction with IAA and does not interact with L-tryptophan and Na-acetyl-L-tryptophan and used by and large[27]. Out of 10 colonies selected from rhizosphere of *Withania somnifera* 6 showed red colour with Salkowaski reagent indicating production of IAA by the organisms. In the present study six of ten isolates were able to produce auxin ranging from 5 to 11 µg ml-1 in the presence of the precursor L-tryptophan in the medium while in the similiar study showed that twelve of fourteen rhizobacteria strains produced 5 to 10 µg ml^{-1} of auxin[15]. It has been reported that IAA production by bacteria can vary among different species and strains, and it is also influenced by culture condition, substrate availability and growth stage. Moreover, isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil[28]. Auxin production by all isolates increased when culture medium supplemented with an IAA precursor; tryptophan which confirm the results of other researcher[29]. The tryptophan increases the production of IAA in *Bacillus amyloliquefaciens* FZB42. In the present study IAA production by the isolates was enhanced from 1.5 to 3 (µg/ml) in the absence of tryptophan and 5 to 11 (µg/ml) in the presence of tryptophan which proves that need of tryptophan as a precursor for IAA production. While, the similar study done by other researcher showed that Azospirillum is able to produce auxins when exposed to tryptophan[30]. Plants inoculated with the rhizobial strain together with Ag^{+} ion and L-tryptophan (Trp), give the highest root dry weight and significantly increase in uptake of N, P and K compared to non-inoculated control plants. tested Fluorescent Pseudomonas isolates for their ability to produce indole acetic acid in pure culture were tested in the absence and presence of L-tryptophan and observed that for both strains, indole production enhanced with increases in concentration of tryptophan[31]. The IAA production was further confirmed by Thin layer chromatography (TLC) in which the (R_f) of the sample isolates was found to be 0.9 corresponding against the standard IAA. Similarly, isolated rhizobia from leguminous plant and tested for the IAA production and its confirmation by thin layer chromatography by R_f value of 0.59 of the isolates followed by 0.57 os the standard IAA[32]. The property of synthesizing IAA is considered as

effective tool for screening beneficial microorganisms suggesting that IAA producing bacteria have profound effect on plant growth[33]. In the present study effect of IAA producers was seen on *Withania somnifera* seeds with significant increase as compared to in germination percentage, root and shoot length as compared to uninoculated seeds. Similarly, it has been reported that Bacterized lentil seeds showed improved plant growth compared to untreated control[21]. A significant difference in percentage of germination was observed compared to control; values ranged between 14.18 to 38.36 %. IAA producing rhizobacterial Isolates exhibiting improved seed germination also supported improved root and shoot length. Seeds coated with rhizobacterial isolates derived from soil representing modern farming practices (MFB-1R-3) showed maximum germination (38.36 %), root length (9.41 cm) and shoot length (2.83 cm). Seed bacterization (or seed coating) has proven to be a method of choice for studying bacterial growth promotion and biological control of plant diseases including pre emergence and post-emergence diseases. In present study, seed treatment with the rhizobacterial isolates significantly improved seed emergence together with plant root and shoot length. Inoculation with IAA producing bacteria induces the proliferation of lateral roots and root hairs. It has been showed that germination rate, roots, shoot growth of plant were increased by IAA as well as PGPR[34]. Therefore these isolates were studied for their effect on plant growth under controlled conditions. There was a significant increase in root and shoot elongation. Among the isolates WSNb3, WSNb5 and WSNb6 were found much effective to show potential increase in root and shoot length. Data obtained from roll towel method experiment and seed germination demonstrated positive effect on plant growth and thus can be considered as plant growth promoter.

CONCLUSION

The isolated organisms were identified as *bacillus spp.* from *Withania somnifera.* It could be concluded that the IAA produced by the organisms could be used as sprays for plant growth promotion. Out of the selected PGPR, WSNb3, WSNb5 and WSNb6 was found to be the best to produce IAA. Co- inoculation of PGPR with other plant growth promoting bacteria can be done for growth promotion. All the rhizobacterial isolates produce only IAA in the culture medium and no other interfering substance which was confirmed by thin layer chromatography (TLC) technique. WSNb3 showed maximum IAA production at all concentrations of Tryptophan. The effect of IAA production was also seen directly on Ashwagandha seeds. The PGPR isolates tend to elevaluate the root and shoot length, seed germination and vigour index as compared to the un-inoculated seeds. These results led to the selection of effective PGPR inoculants that are beneficial for the growth of medicinal plants. These inoculants if applied directly to the plant or its parts can be effective in growth stimulation.

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