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RESEARCH ARTICLE

INDOLE ACETIC ACID PRODUCTION FROM *PSEUDOMONAS FLUORESCENS* AND ITS EFFECT ON ROOT ELONGATION OF *VIGNA RADIATA*

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ABSTRACT

Rhizospheric soil samples were collected from in different garden samples in Chidambaram. Among 7 isolates 1 strain was developed deep pink colour, which was selected as potential strain for further study. It was showed highest OD value at 530nm. It was identified as *Pseudomonas fluorescens* was found to produce IAA 2.5µg/ml. The maximum production of 3.7µg/ml was observed at pH 7 and minimum at pH 10 (1.9µg/ml). Maximum production of 3.3µg/ml was observed at 30°C which seemed to be the most favorable and minimum was observed at 40°C (1.5µg/ml). The effect of Nacl on the IAA production maximum 2.9µg/ml (0.2%) and minimum 0.8µg/ml (1.0%), different carbon sources maximum 3.9µg/ml in glucose and minimum fructose (2.3µg/ml), different nitrogen sources on IAA maximum 3.8µg/ml in yeast extract and minimum potassium nitrate were observed. Influence of tryptophan on the IAA production maximum 3.5µg/ml at 1.0% and minimum at 3.0% (2.1µg/ml) were noted. *Pseudomonas fluorescens* in mass scale with optimized parameters, produced the highest IAA at 8th day of incubation (4.6µg/ml) On further incubation the amount of IAA was found to be reduced. The TLC done for the crude IAA compound showed a R_f-value of 0.88. The purified and standard showed same R_f values. The seed germination test revealed 100% germination in IAA treated seeds and only 76% germination was observed in control seeds. In the present observation the average *Vigna radiata* root length developed in 50 seedlings was estimated in both IAA treated and control seedlings. The average root length was 42mm in IAA treated seedlings where as it was only 28mm in control seedlings.

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INTRODUCTION

The current global scenario firmly emphasizes the need to adopt eco-friendly agricultural practices for sustainable agriculture. Chemical agriculture has made an adverse impact on the health-care of not only soil but also the beneficial soil microbial communities and the plants cultivated in these soils. This eventually has lead to a high demand for organic produce by the present-day health conscious society and sporadic attempts are being made by farmers all over the world to detoxify the land by switching over to organic farming dispensing with chemical fertilizers, pesticides, fungicides and herbicides etc.,

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Indole acetic acid (IAA) is one of the most physiologically active auxins. The term plant hormone is a naturally occurring chemical substance which is an integral part of plant metabolism and in small concentrations can activate or depress any developmental process in that plant. Plant hormones are used extensively in agriculture, horticulture, and biotechnology to modify plant growth and development. Hormones regulate or influence a range of cellular and physiological processes including cell division, cell enlargement, cell differentiation, flowering, fruit ripening, movement (tropisms), seed dormancy, seed germination, senescence, leaf abscission and stomatal conductance. Some species like *Azospirillum sp*., *Enterobacter sp*., *Azotobaeter sp*., *Pseudomonas sp*., *Alcaligene sp.*, *Serratia sp*., cyanobacteria and sulfur oxidizing bacteria have shown to encourage plant growth via plant hormone release.

In 1880 Charles Darwin proposed that some plant growth responses are regulated by a matter which transmits its effects from one part of the plant to another (Darwin). Several decades later, this matter, termed aux in (from the Greek auxein which means to grow), was identified as indole-3-aceticacid (IAA) (Kogl and Kostermans, 1934 and Went and Thimann, 1937). IAA has since been implicated in virtually all aspects of plant growth and development (Woodward and Bartel, 2005). In recent years, advancement in understanding the IAA signaling path way in plants has been truly spectacular. The role of IAA in bacteria has not thus far been investigated in such detail. Undoubtedly, the advancement in plant IAA signaling has also intensive research on the various aspects of bacterial IAA synthesis, including its role in bacteria plant interactions. A First class of factors influencing IAA biosynthesis in diverse bacteria is related to In *Az.brasilence* IAA production and expression of the key gene ipdC have been shown to be increased under carbon limitation, during reduction in growth rate and under acidic pH (Ona *et al*., 2003, 2005 and Vande Broek *et al*., 2005). The phytohormone indole-3-acetic acid (IAA) is the most commonly occurring naturally produced auxin and the most thoroughly studied plant growth regulator. IAA directs several aspects of plant growth and development (Pattern and Glick, 2002), including the induction and regulation of a variety of processes: e.g., cell division, root extension, vascularization, apical dominance and tropisms (Bartel, 1997). The effects of IAA on plant root tissue are concentration dependent and can be species specific. Responses to increasing IAA concentrations revealed an advance from the stimulation of primary root tissue to the development of lateral and adventitious roots and finally end up to the complete cessation of root growth if concentration exceeds (Leveau and Lindow, 2005).

Initially, *Azotobacter* and *Azospirillum* were believed to promote plant growth due to their ability to fix dinitrogen. Later, it was known that other plant growth stimulating hormones such as IAA was also involved (Kennedy, 1998). The use of P-solubilizing bacteria was reported to increase plant growth in some cases, but in other cases it was not. It indicated that other mechanisms may involve in growth response (De Freitas *et al*., 1997). One of the most important ways that those bacteria affect growth and development is by producing Indole-3-acetic acid (IAA) that this hormone is led to plant root system development and subsequently nutritional uptake by implant is increased. As plant roots grow through soil, they release water-soluble compounds such as amino acids, sugars and organic acids that supply food for the microorganisms. In return, the microorganisms provide nutrients for the plants. All these activities makes the rhizosphere the most dynamic environment in soil. Because roots are underground rhizosphere activity has been largely overlooked. The Rhizosphere is a center of intense biological activity due to the food supply provided by the root exudates. Hence the present study is an IAA production from a bacterial strain isolated from garden soil in Chidambaram where lush growth of varied plants was observed.

MATERIALS AND METHODS

Collection of samples

Rhizospheric soil samples were collected from a garden in Chidambaram in January 2017 for the isolation of IAA producers. To isolate IAA producing strains from rhizospheric soil samples, samples were serially diluted with 9ml distilled water blanks, 0.1ml of each dilution (10^{-3}, 10^{-4} and10^{-5}) were spread plated on Luria agar plates with L-tryptophan and incubated at 30°C for 72 hrs.

Screening of indole acetic acid (IAA) production

Screening was done by using Luria broth supplemented with L-tryptophan

Media composition (g/L)

Tryptone-10.0
Yeast extract-5.0
L-tryptophan-1.0
Nacl-5.0g
Distilled water-1000ml
pH-7.0-7.5

7 strains were screened for IAA production. A loopful of culture was inoculated in 10ml of Luria broth supplemented with L-tryptophan and incubated for 72hrs at 30°C. Then, the culture was centrifuged at 10,000xg for 10min., and the supernatant was collected. One ml of supernatant was allowed to react with 2ml of Salkowsky reagent (1ml of 0.5M $FeCl_3$ in 50ml of 35% $HClO_4$) at 30°C for 30 minutes. Pink colour development indicated the presence of IAA (Patten and Glick, 1996).

Estimation of IAA

The potential strain culture was centrifuged at 13,000x g for 10min. and the supernatant was collected. The presence of IAA was measured in a spectrophotometer by adding 2ml of Salkowsky reagent to 1ml of supernatant, incubated for 30min. The optical density was read at 530nm. The recorded OD values were plotted in a standard curve prepared from commercially available IAA (Khan and Doty, 2009) and their concentration was calculated.

Identification of potential strain

Base on the result obtained on IAA value as estimated above potential strain was sleeved. The potential strain was identified according to the method described by Bergeys manual (Buchanan *et al*., 1974).

Optimization of IAA production

Factors like pH (5-10), temperature (25°C- 40°C), sodium chloride (0.2-1.0%), carbon sources (glucose, sucrose and fructose) and nitrogen sources (peptone, yeast extract, beef extract and ammonium nitrate) were optimized and observed for a period of 5 days.

Mass scale production

Mass scale production was done in shake flasks (250ml in 500ml flask). Optimized parameters viz., 30°C, pH 7, 0.2% Nacl, glucose 1% and yeast extract 0.5% were kept and the media in four flasks were kept for incubation. IAA production was estimated at an interval of 2 days for a period of 10 days.

Extraction of IAA

Extraction was done following the procedure of Ahmad et al., 2005.

Bacterial cells were separated from the supernatant by centrifugation at 10,000xg for 30min., supernatant was acidified to pH 2.5 to 3.0 with 1N HCl and extracted twice with ethyl acetate at the rate of double the volume of the supernatant, which was then evaporated to dryness in a rotary evaporator at 40°C. The extract was dissolved in methanol and kept at -20°C.

Thin layer chromatography

Purified sample and standard IAA (10-20µg) were placed on TLC plates (Slica gel G thickness 0.25mm, Merck) with a solvent system (ethyl acetate and hexane, 2:8) and then sprayed with Salkowski reagent. Rf value was calculated (Morales *et al*., 2003).

Effect of IAA on germination of seeds

The positive effect of IAA on germination of seeds was tested by modifying the method of Anis *et al*. (2010). 50 green gram seeds were soaked overnight in 15-20ml of IAA containing broth obtained through mass scale culture. The effect of IAA on germination was compared with that of control i.e. seeds soaked in Luria broth alone.

Effect of IAA on root elongation

Both IAA treated and the control seeds were sow in two different pots containing 3kg of garden soil. Regular watering was done for a period of two weeks. The plants were plucked carefully after profusely watering the pots. The length of root developed in both the sets was compared.

RESULTS

Rhizospheric soil samples were collected from in different garden samples in Chidambaram. The samples were plated Luria agar supplemented with L-tryptophan. The maximum density of the bacteria was found to be $2.1x10^4$CFU/g. 7 isolates obtained from different rhizospheres of plants were screened with L-tryptophan containing medium for the productivity of IAA. 1ml of culture filtrates was allowed to react with 2ml of Salkowsky reagent at 30°C for incubated for 30min. At the end of the incubation, pink colour was developed which indicated the presence of IAA. Among 7 isolates 1 strain was developed deep pink colour, which was selected as potential strain for further study. It was showed highest OD value at 530nm. It was identified as *Pseudomonas fluorescens* (Table 1). *Pseudomonas fluorescens* was found to produce IAA 2.5µg/ml.

Table1: Physiological and biochemical characteristics of *Pseudomonas fluorescens*

Gram reaction	_
Shape of the cell	R
Spore	_
Motility	_
Adonitol	+
Arabinose	+
Sorbitol	+
Sucrose	+
Starch	+
Gelatine	+
Fat	+
Catalase	+
Pigment	+
Indole	+

In the present study influence of pH on the IAA production was carried out at different pH. The maximum production of 3.7µg/ml was observed at pH 7 and minimum at pH 10 (1.9µg/ml) (Fig.1).

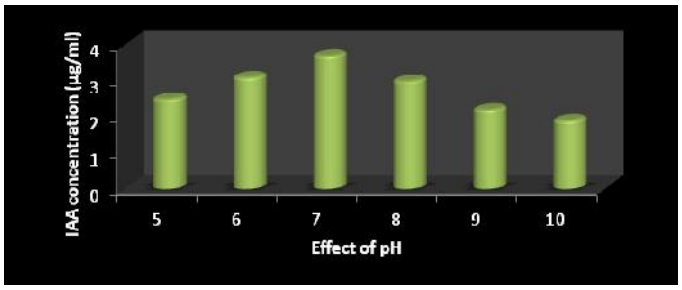

Fig:1 Effect of pH on IAA production

In the present observation, the optimum temperature required for the maximum IAA production was tested. Maximum production of 3.3µg/ml was observed at 30°C which seemed to be the most favorable and minimum was observed at 40°C (1.5µg/ml) (Fig:2).

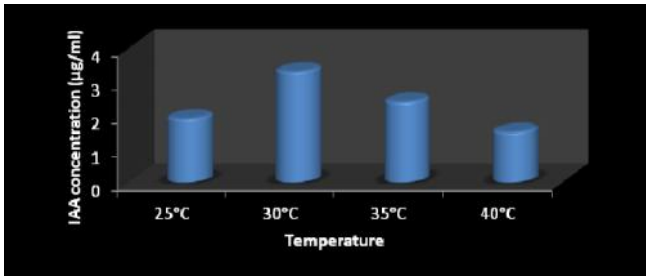

Fig. 2 Effect of temperature on IAA production

In the present investigation, the effect of Nacl on the IAA production was carried out at different Nacl concentration. The maximum production of 2.9µg/ml was observed at 0.2% and minimum at 1.0% (0.8µg/ml) (Fig. 3).

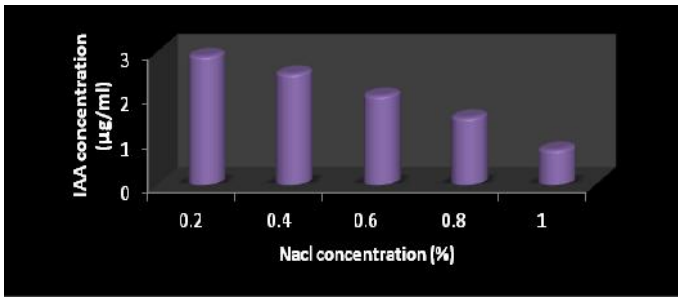

Fig. 3. Effect of Nacl concentration on IAA production

In this study, the different carbon sources were tried. The maximum production of 3.9µg/ml was observed in glucose and minimum was found at fructose (2.3µg/ml) (Fig. 4).

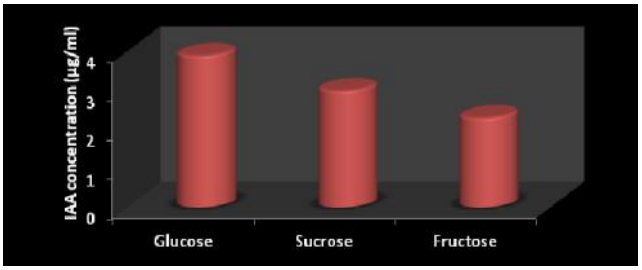

Fig. 4. Effect of carbon sources on IAA production

In this work, the effect of different nitrogen sources on IAA production was carried out. The maximum production of 3.8µg/ml was observed in yeast extract and minimum was with potassium nitrate (2.4µg/ml) (Fig. 5).

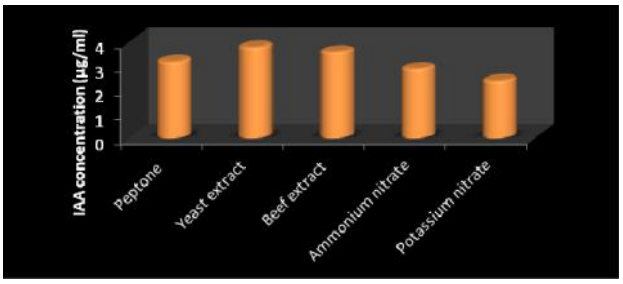

Fig. 5. Effect of nitrogen sources on IAA production

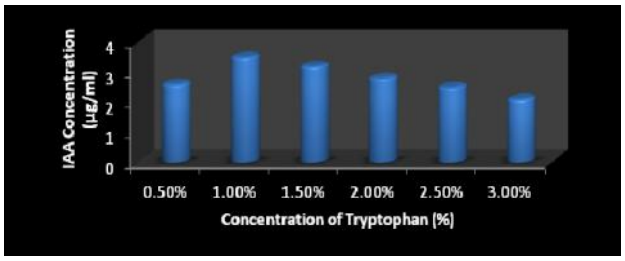

Fig. 6. Effect of concentration of Tryptophan on IAA production

In the present study influence of tryptophan on the IAA production was carried out at different concentration. The maximum production of 3.5µg/ml was observed at 1.0% and minimum was found at 3.0% (2.1µg/ml) (Fig. 6). In the present observation, *Pseudomonas fluorescens* in mass scale with optimized parameters, produced the highest IAA at 8th day of incubation (4.6µg/ml) (Fig. 7). On further incubation the amount of IAA was found to be reduced.

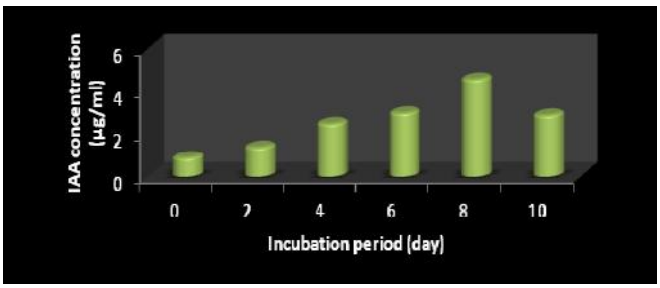

Fig. 7. Effect of incubation period on IAA production

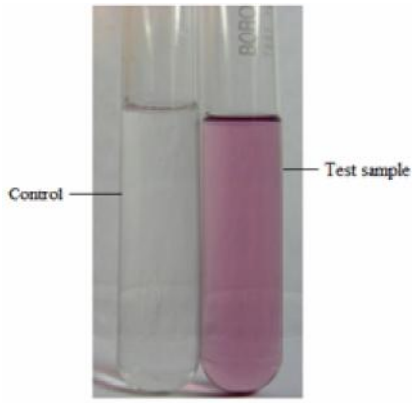

Fig. 8. IAA production of *Pseudomonas fluorescens*

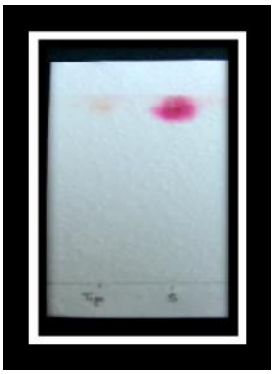

Fig. 9. TLC of standard and crude IAA S- stands for Standard IAA, Tip- stands for Crude sample

The TLC done for the crude IAA compound and standard IAA sprayed with Salkowski reagent showed a R_f-value of 0.88. The purified and standard showed same R_f values. The seed germination test revealed 100% germination in IAA treated seeds and only 76% germination was observed in control seeds. The average root length developed in 50 seedlings was estimated in both IAA treated and control seedlings. The average root length was 42mm in IAA treated seedlings where as it was only 28mm in control seedlings.

Fig. 10. Effect of root elongation without IAA

Fig. 11. Effect of root elongation with IAA

Fig. 12. Plant growth promoted by Crude IAA

Fig. 13. Control pot

DISCUSSION

IAA is an important phytoharmone and is the most common natural auxin found in plants. Root tissues are especially sensitive to fluctuating concentrations of IAA and the development of root system seems to be greatly affected by exogenous sources of this plant growth regulation including microbial (Tanimoto, 2005). It is a common sight in any garden that a few plants have lush growth where as others are not. The reason may be existence of healthy microenvironments or niches below the soil surface especially around the roots of the plants known as rhizosphere area. Having this in mind the soil samples were collected from subsurface soil of lush greeneries. In one such sample density of IAA producers were at the level of $2.1x10^4$CFU/g. This sample was selected for further study. Patten and Glick, (1996) also opined that IAA producers are widespread in nature.

The plant growth regulator indole acetic acid (IAA) has long been postulated to play a role in one or more aspects of nodule growth and development and the detection of increased levels of IAA in nodule tissue supports this hypothesis (Kittell Barbara, 1989). The most potential strain was identified as *Pseudomonas fluorescens*. The ability to produce IAA seems to be widespread among microbes. Such microbes include rhizobia (Jones *et al*., 1982), mycorrhizal fungi and *Pseudomonads* (Parikryl *et al*., 1985 and Loper and Schroth, 1986). *Pseudomonads* are aggressive colonizers of rhizoshpere of various plants (Cartwright *et al*., 1985). Similarly*Pseudomonas fluorescens* AK1 and *Pseudomonas aeruginosa* AK2 showed the best plant growth-promoting activity. In the present study among various strains tested the selected strain was found to produce 2.5µg/ml of IAA. Microbes need certain set of physico-chemical parameters for the selective production of compounds in appreciable quantity.

In the present study, ideal parameters for maximum proudction of IAA were tested. Regarding pH, pH 7 was found to be ideal at which 3.7 µg/ml was produced. Huddedar (2000) also reported pH 7.0 as optimum in *A.baumanii* A16 and *Acinetobacter* A18 strain. Regarding temperature 30^{o}C was found to suitable in the present study. Apine and Jadhav, (2011) also found 30^{o}C was suitable for IAA production respectively in *Pantoea agglomerans* pvm and *Pseudomonas spp.* In the present study 0.2% of NaCl resulted in higher concentration of IAA (2.9µg/ml). In a mangrove soil isolate *A.brasilense* 1% NaCl was found to be optimum (Ravikumar *et al*., 2004). As the strain was isolated from garden soil, the isolate might have preferred low percentage of Nacl.

Apart from 1% tryptophan three other carbon sources were tested in the present study. Glucose was preferred by the strain. When various concentration of tryptophan was tested 1% was found to be ideal. On further increase of tryptophan, IAA was found to decrease in quantity. However when glucose was added with 1% tryptophan the quantity of IAA produced was more compared to that of 1% tryptophan alone. However in the present study tryptophan was not excluded in any experiment. The study proved that excess of tryptophan is detrimental for IAA production and addition of secondary carbon source enhanced the production. Narayana *et al*. (2009) also observed as 1% glucose as the preferable substrate for IAA production.
The quantity of IAA produced in the present study was 4.6µg/ml at optimized conditions. Prikryl *et al.* (1985) observed 0.01-3.93mg/l of auxin production. Compared to this the amount produced in the present study was more. However Patil, (2011) reported more, in 3 strains of *Azobacter.* The range of IAA production in *Azotobacter* isolates without tryptophan was 2.68-10.80mg/ml. A significant increase in the production of IAA was recorded in the presence of 1, 2 and 5 mg/ml of tryptophan, i.e. 1.47-11.88 mg/ml, 5.99-24.8 mg/ml and 7.3-32.8 mg/ml respectively. These studies along with the present study showed the existence of wide variation in level of IAA production among microbes.

A number of free-living bacteria have the ability to fix dinitrogen and increase nitrogen availability for plant. IAA produced by bacteria improves plant growth by increasing the number of root hairs and lateral roots (Okon and Kapulnik, 1986). Microbical biosynthesis of IAA in soil is enhanced by tryptophan from root exudates or decaying cells (Frankenberger and Arshad, 1991 and Benizri *et al*., 1998). Our TLC findings are in agreement with reports by other scientists (Xie *et al*., 1996). In the present study TLC of the crude IAA compound and standard IAA sprayed with Salkowski reagent showed R_f-value of 0.88. In the present study the seed germination test revealed 100% germination in IAA treated seeds and 76% germination in IAA non-treated or control seeds. The average root developed in 50 seedlings were estimated in both IAA treated and control seedlings. The average root length was 42mm in IAA treated seedlings where as it was 28mm in control seedlings. Several researchers reported the positive effect of Pseudomonads on growth characteristics of different crop plants (Glick, 1995; Shishido and Chanway, 2000 and Yao *et al*., 2008). *Pseudomonads* isolates used in pot experiment showed positive effects on growth and yield of rice. However, they were different in their potential to enhance plant growth. In a study of Kloepper *et al*., (1989), inoculated different strains of *Pseudomonas* to the rice and an increase in the yield production ranging from 3-160% were obtained. The study proved that IAA could play a role in seed germination as well as in development of roots.

There are numerous soil microflora involved in the synthesis of auxins in pure culture and soil (Barazani and Friedman, 1999). Some microorganisms produce auxins in the presence of asuitable precursor such as L-tryptophan. The effects of auxins on plant seedlings are concentration dependent, i.e. low concentration may stimulate growth while high concentrations may be inhibitory (Arshad and Frankenberger, 1991). Different plant seedlings respond differently to variable auxin concentrations (Sarwar and Frankenberger, 1994) and type of microorganisms. However, there are some microorganisms that do interact with specific plants. These interactions can be pathogenic, symbiotic, harmful, saprophytic or neutral.

Interactions that are beneficial to agriculture include mycorrhizae, legume nodulation and production of antimicrobial compounds that inhibit the growth of pathogens. Rhizosphere microorganisms produce vitamins, antibiotics, 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 mineral nutrients, synthesis of vitamins, amino acids, auxins, cytokinins and gibberellins which stimulate plant growth and antagonism with potential plant pathogens through competition and development of amensal relationships based on production of antibiotics.

The action and interaction of some growth regulators like auxins regulate most of the physiological 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 the ability to synthesize phytohormone is widely distributed among plant associated bacteria. 80% of the *bacteria* isolated from plant Rhizosphere are to produce IAA.

The findings of the present investigation highlighted that IAA producing bacteria *Pseudomonas fluorescens* from local soil could be easily isolated and may be exploited after strain improvement for local use. However, further studies using IAA mutant strains of these isolates are needed to explore the exact contribution of IAA production in the promotion of plant growth as well as the contribution of other PGP traits.

Conclusion

The seed germination test revealed 100% germination in IAA treated seeds and 76% germination in IAA non-treated or control seeds. The average root developed in 50 seedlings was estimated in both IAA treated and control seedlings. The average root length was 42mm in IAA treated seedlings where as it was only 28mm in control seedlings. Some microorganisms produce auxins in the presence of a suitable precursor such as L-Tryptophan. They effects of auxins of plant seedlings are concentration dependent i.e. low concentration may stimulate growth while high concentrations may be inhibitory. Different plant seedlings respond differently to variable auxin concentrations and type of microorganisms.

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