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**Research Article**

# Population density and *in vitro* characterization of selected PGPRs from tobacco rhizosphere soils

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**ABSTRACT:** Present study revealed that PGPRs such as *Bacillus*, *Pseudomonas, Trichoderma* and *Streptomyces* spp. present predominantly in tobacco rhizosphere soils. Population density of PGPR was found to be higher in northern light soils (NLS) followed by Karnataka light soils (KLS) than in southern light soils (SLS) and traditional black soils (TBS) whereas lowest was recorded in chewing tobacco soils. Moreover, among the PGPRs, bacterial PGPRs such as *B. subtilis* and *P. fluorescens* were found to be higher than actinomycetes (*Streptomyces* spp.) and fungi like *T. viride*. Similarly, of the bacterial PGPRs, *P. fluorescens* population was the highest (11.98 x$10^5$) in NLS while *B. subtilis* was the least (5.99 x$10^5$ cfu/gm soil dry wt.) in chewing tobacco. The population density of *T. viride* recorded least in chewing tobacco soils of West Bengal and Tamil Nadu (3.18 x $10^3$ and 2.77 x $10^3$ cfu/gm soil dry wt., respectively). The population density of PGPR strains positively coincided with soil nutrients like total organic matter, phosphorous and potassium contents. The results on biochemical characterization indicated that most of the strains were efficient in hydrolyzing starch, gelatin and casein. Among the different carbon and nitrogen sources tested, maltose and potassium nitrate, respectively were the good supplementary sources. Of the different amino acid and vitamin sources evaluated, alanine and riboflavin, respectively were found to be limiting substrates for maximum growth of PGPR strains. Antagonistic activity of *Streptomyces* sp. against *Pythium aphanidermatum* showed 37.52 per cent of inhibition over control.

**KEY WORDS:** Biocontrol, PGPR, population density, tobacco

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## INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is a low volume and high value commercial crop, belongs to the family Solanaceae in which nicotine has been identified as the active principle compound. The tobacco leaf is affected by various pests and diseases which in turn reduce the quality of leaf.

The increasing use of chemical fertilizers for higher crop productivity is adversely affecting quality of soil and tobacco leaf. Application of chemical biocides and synthetic fertilizers is an effective technique to manage plant diseases, but it is controversial because of its higher costs and adverse environmental effects (Raveendra *et al*., 2007). Contrary to the chemical fertilizers, organic manures and bioinoculants are less expensive and increase productivity without harming the environment. It is believed that soil microorganisms play a major role in nutrient cycling and plant growth in contrast to conventional agriculture. These criteria can be achieved through the application of microbial inoculants like PGPRs (Subhashini, 2013), because these microorganisms are known to possess vast range of capabilities by producing growth promoting substances, enzymes, vitamins, organic aids, bioactive compounds, enhancing the plant nutrients, biological $N_2$ fixation and phosphorous/potassium-solubilization for crop protection against stress and diseased conditions. Plant growth promoting rhizobacteria (PGPR) have been proved as efficient biocontrol agents in controlling various plant diseases, besides, enhancing the plant growth significantly (Subhashini and Padmaja, 2011).

The present study envisages detailed examination of the population density of different known PGPR strains in tobacco rhizosphere soil samples and the antagonistic activity of PGPRs by testing against damping-off causing pathogen, *Pythium aphanidermatum* in tobacco nurseries.

## MATERIALS AND METHODS

Soil samples from different tobacco growing zones of India were collected into sterile bags from 6-10 cm depth due to high microbial activity. The samples were drawn from Southern light soils (SLS) of Prakasam district, Kandukur (A.P.), Northern light soils (NLS) of West Godavari

district, Jeelugumilli, (A.P.), and Traditional black soils (TBS) of East Godavari district, Katheru. (A.P.), Karnataka Light Soils (KLS) of Mysore district, Hunsur, Karnataka, Chewing tobacco growing zones of Tamil Nadu (Vedasandur) and West Bengal (Dinhata) for the estimation of nutrient contents, enumeration of different PGPRs during 2005-2010. These samples were allowed to air dry at room temperature and various parameters like soil pH, total organic carbon (Walkley and Black, 1934), available phosphorous and available potassium (Jackson, 1973) were determined. The population density of different PGPRs such as *Bacillus*, *Pseudomonas*, *Trichoderma* and *Streptomyces* species were enumerated in the above soil samples using nutrient agar (NA), King's B, *Trichoderma* selective medium (TSM) and casein nitrate agar (CNA) media respectively.

The pathogen, *Pythium aphanidermatum* was isolated from infected tobacco seedlings of tobacco seed beds at Central Tobacco Research Institute (CTRI), Rajahmundry, Andhra Pradesh, India. Tobacco seeds @ 0.5g /$m^2$ bed were sown thickly in pots containing farm soil collected from nursery site under glasshouse conditions. After sowing, the pots were kept under shade and watered daily to favour the incidence of damping-off. After 15 days, seedlings showing damping-off symptoms were collected and the pathogen, *P. aphanidermatum* (Edson) Fitzp. was isolated by tissue segment method (Rangaswami,1958) on Potato Dextrose Agar medium (PDA). It was purified by single hyphal tip method and maintained in potato dextrose agar slants.

Enumeration and isolation of different PGPRs in the soil samples were performed by serial dilution plate technique. Morphological characterizations such as Gram's staining, endospores and motility test and biochemical characterizations such as pigment production, starch hydrolysis, casein hydrolysis, catalase test, nitrate reduction, indole production, gelatin hydrolysis, and hydrogen sulphide production were carried out (Williams and Wilkins, 1994). Influence of abiotic factors such as pH (4.0 to 8.5) and temperature (5 to 40°C) and various nutrient factors such as carbon, nitrogen, amino acid and vitamin sources on growth of various PGPRs were carried out. Five different carbon compounds such as glucose, fructose, maltose, sucrose, and starch and three nitrogen compounds such as ammonium nitrate, potassium nitrate and casein hydrolysate were added by replacing starch and potassium nitrate; respectively in the basal medium. On the other hand, sources of amino acid like alanine and methionine and vitamins such as thiamine and riboflavin were selected and supplemented extra in the basal medium to enhance the growth potential of PGPRs. The inoculated plates were incubated for 10-15 days depending upon the nature of experiment to record the growth rate of PGPRs (Ponmurugan *et al*., 2007).

Interaction between PGPR isolates and *P. aphanidermatum* was studied by following the method of dual culture technique. In the case of dual culture study, a mixture (50:50 %) of PDA and NA /King's B/TSM/CNA media containing plates were inoculated with *P. aphanidermatum* as well as PGPRs strains on diametrically opposite points (Huang and Hoes, 1976). Radial growth of the pathogen and antagonist were measured at 24 hrs interval. Radial growth was measured periodically and the per cent inhibition was calculated.

## RESULTS AND DISCUSSION

Soil fertility and plant nutrition play a significant role in influencing the yield and quality of tobacco leaf. Tobacco plant requires adequate and continuous supply of all the essential plant nutrients at optimum level in a balanced manner during the growing period and a rapid decline in the supply of these nutrients in the soil as the crop approaches maturity. A balanced and desirable tobacco growth can only be achieved with an adequate and well timed supply of nutrients (Krishnamurthy and Deo Singh, 2002). Many beneficial microorganisms promote plant growth by mutualistic or symbiotic relationship by fixing atmospheric nitrogen or mobilizing phosphorous or solubilizing potassium to a greater extent (Subhashini, 2011). It has been observed that the rhizosphere is a region of dynamic process initiated by root exudations, release of organic nutrients and is influenced by a host of factors like soil features, environmental conditions, cultural practices and soil microbial interactions (Baby *et al.,* 2002). Among the beneficial mutualistic with non-symbiotic microorganisms, *Bacillus subtilis, Pseudomonas fluorescens, Trichoderma viride* and *Streptomyces* species are very important in tobacco plant growth in terms of enhancing the yield potential and maintaining soil health. The results revealed that the total number of various PGPRs population in tobacco soil samples was found to be higher in NLS followed by KLS than in SLS and TBS, whereas lowest population density of PGPR was recorded in chewing tobacco soils. Moreover, among the PGPRs, bacterial PGPRs such as *B. subtilis* and *P. fluorescens* were found to be higher than actinomycetes (*Streptomyces* spp.) and fungal PGPRs like *T. viride*. Similarly, of the bacterial PGPRs, *P. fluorescens* was the highest (11.98 x$10^5$) population in the soils of NLS while *B. subtilis* was the least (5.99 x$10^5$ cfu/gm soil dry wt.) population. The population density of *T. viride* was recorded least in chewing tobacco soils of West Bengal and Tamil Nadu (3.18 x $10^3$ and 2.77 x $10^3$ cfu/gm soil dry wt., respectively).

The population density of PGPR strains positive-

ly coincided with soil nutrients like total organic matter, phosphorous and potassium contents (Table 2). Moreover, the population density of PGPRs was found to be more in rhizosphere than in non-rhizosphere soil samples collected from tobacco fields. The same trend was registered in population density which was found to be maximum in soil samples obtained from NLS followed by KLS and least with chewing tobacco soils of Tamil Nadu and West Bengal. The soil parameters like soil reaction (pH), Organic carbon (%), Available phosphorus (ppm) and Available K (ppm) were varied in varying levels between tobacco planting zones, however, there was a strong relationship between these parameters and population diversity of PGPRs (Table 1). Similar observations were reported in tea soils by Baby *et al.,* (2002) and Ponmurugan *et al*., (2011) who observed a correlation between beneficial microorganisms like nitrogen fixers, phosphate solubilizers, antibiotic producing microorganisms (actinomycetes) and biocontrol agents and nutrients in the soil.

Different strains of PGPRs were isolated from tobacco soil samples. Further, morphological and biochemical characteristics of the selected PGPR strains based on their bio control activity against *P. aphanidermatum* along with the check (*Pseudomonas fluorescens*) obtained from Tamil Nadu Agricultural University were studied and results were presented in Table 1 and Table 2. Morphological characterization showed that the isolates are Gram's positive organisms. The cells were coiled rods and the endospores were free to tight in nature. The results of biochemical characterization indicated that most of the strains were efficient in hydrolyzing starch, gelatin and casein. Indole production was strictly negative for some strains but catalase test was positive to the rest of strains. Production of hydrogen sulphide and nitrite reduction showed positive result in majority of the isolates (Table 3). The results coincided with the report of Ravel *et al*. (2000) and Ponmurugan *et al*. (2011).

The growth of PGPR strains in the basal medium adjusted with different pH and nutrient sources revealed that a better growth was recorded at pH 5.2 to 5.7 (Table-4). This pH level may be correlated with the soil pH. The optimum temperature for the growth of PGPR strains was 25°C -28°C (Table-4). Among the different carbon and nitrogen sources tested, maltose and potassium nitrate, respectively the good supplementary sources. Of the different amino acid and vitamin sources evaluated, alanine and riboflavin, respectively were found to be limiting substrates for maximum growth of PGPR strains (Table 4). Production of an array of antifungal metabolites has been known to be influenced by components of medium and cultural conditions such as pH, temperature, carbon, nitrogen and other sources (Augustine *et al*., 2004).

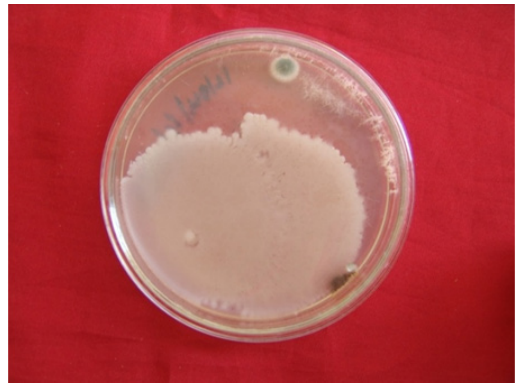

**Fig. 1. Antagonistic activity of *Bacillus* sp. against *Pythium aphanidermatum.***

The antagonistic potential of various PGPR strains was studied against damping-off disease causing pathogen namely *P. aphanidermatum*, which revealed a remarkable percentage of inhibition of pathogen growth (Fig. 1 and Fig. 2). These results coincided with the report of Subhashini and Padmaja (2009) who observed the inhibition zone of *P. aphanidermatum* with Pseudomonas fluorescens and *Streptomyces lavendula* (Subhashini 2010). The growth inhibition of pathogen is due to the production of secondary metabolites by the antagonists (Thangapandian *et al*., 2007). According to Demain and Fang (1995), the most widely accepted theory is that antibiotics are used to compete with other organisms in nutrient depleting environment (Ravel *et al*., 2000 Augustine *et al*., 2004) and the production of exopolysaccharide compounds, diffusible pigments and enzymes such as lipase, caseinase, gelatinase, cellulase and amylase by the antagonists (Ponmurugan *et al*., (2011). In the present study, most of the strains of PGPRs were found to be potential antagonists against damping-off pathogen. Based on these results, it can be further inferred that the isolated PGPR strains can be used as soil inoculants to prevent the growth of soil-borne pathogens like *P. aphanidermatum* in tobacco soils.

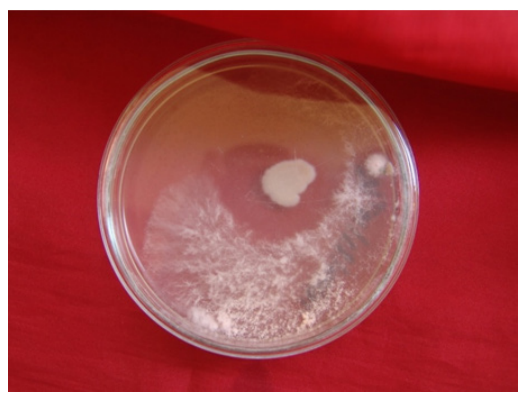

**Fig. 2. Antagonistic activity of *Streptomyces* sp. against *Pythium aphanidermatum.***

**Table 1. Effect of PGPR antagonists on the growth of *Pythium aphanidermatum in vitro***

| Strains of various PGPRs | Radial growth of pathogen(cm) | % inhibition over control |
|---|---|---|
| *Bacillus* | 6.7 | 27.17 |
| *Pseudomonas* | 6.7 | 27.17 |
| *Streptomyces* | 6.3 | 31.52 |
| *Trichoderma* | 7.5 | 18.47 |
| *Pseudomonas* - (TNAU)-check | 5.6 | 39.13 |
| Control | 9.2 | |
| S.E.M 0.08 | | |
| C. D (*P* = 5 % 0.25) | | |
| C.V% 1.97 | | |

**Table 2. Population density of various PGPRs and nutrient status in tobacco soils**

| Name of the soil growing tobacco | Population (cfu/gm soil dry wt.) | | | | Soil (pH) | Organic carbon (%) | Available phosphorus (ppm) | Available K (ppm) |
|---|---|---|---|---|---|---|---|---|
| | BS ($10^5$) | PF ($10^5$) | T V ($10^3$) | SS ($10^4$) | | | | |
| Traditional Black soils | 07.30 | 09.90 | 04.70 | 07.50 | 8.00 | 0.50 | 14.80 | 325.00 |
| Northern Light Soils | 09.20 | 11.90 | 06.10 | 08.60 | 7.30 | 0.30 | 08.70 | 248.00 |
| Southern Light Soils | 07.00 | 10.00 | 04.20 | 05.30 | 7.60 | 0.30 | 04.90 | 265.00 |
| Karnataka Light Soils | 09.10 | 11.90 | 05.80 | 07.70 | 5.70 | 0.20 | 08.00 | 238.00 |
| Chewing tobacco (West Bengal) | 06.40 | 09.80 | 03.20 | 04.10 | 5.60 | 0.60 | 49.80 | 178.00 |
| Chewing tobacco (Tamil Nadu) | 05.90 | 09.80 | 02.80 | 04.00 | 8.20 | 0.40 | 21.90 | 240.00 |
| S.Em± | 00.16 | 00.23 | 00.09 | 00.14 | 0.09 | 0.02 | 00.43 | 010.29 |
| CD (*P* = 5%) | 00.49 | 00.73 | 00.30 | 00.44 | 0.29 | 0.06 | 01.35 | 032.41 |
| **CV%** | **03.63** | **03.82** | **03.76** | **03.89** | **2.25** | **9.10** | **04.11** | **007.15** |

**Table 3. Morphological, physiological and biochemical characterization of various PGPRs**

| Parameters | Strains of various PGPRs | | | |
|---|---|---|---|---|
| | *Bacillus* | *Pseudomonas* | *Streptomyces* | *Trichoderma* |
| Cell morphology | Coiled rods | Rods | Spiral spore chains, Rods, Coiled rods | Cluster of spore chains |
| Gram's staining | + | + | + | - |
| Pigment production | + | + | ++ | - |
| Starch hydrolysis | + | - | + | ++ |
| Casein hydrolysis | + | + | + | + |
| Catalase test | + | ++ | ++ | + |
| Nitrate reduction | ++ | ++ | ++ | ++ |
| Indole production | + | + | + | - |
| Gelatin hydrolysis | + | + | ++ | - |
| Hydrogen sulphide production | ++ | ++ | ++ | - |
| Presence of endospores | ++ | ++ | ++ | - |
| Nature of endospores | Free cells | Tight cells | Tight cells | Free |

++ Positive; + Moderate; - Negative reaction

**Table 4. Effect of abiotic and nutrient factors on the growth of PGPR**

| Parameters | Strains of various PGPRs | | | |
|---|---|---|---|---|
| | *Bacillus* | *Pseudomonas* | *Streptomyces* | *Trichoderma* |
| Optimum pH | 05.3 | 05.2 | 05.7 | 05.4 |
| Optimum temperature (°C) | 25.0 | 28.0 | 27.0 | 27.0 |
| Glucose* | + | + | + | ++ |
| Fructose* | + | ++ | + | + |
| Maltose* | ++ | ++ | ++ | ++ |
| Sucrose* | + | + | - | - |
| Starch* | ++ | ++ | ++ | ++ |
| Ammonium nitrate** | + | + | + | + |
| Potassium nitrate** | ++ | ++ | ++ | ++ |
| Casein hydrolysate** | + | + | ++ | + |
| Alanine# | ++ | ++ | ++ | ++ |
| Methionine# | + | + | + | + |
| Thymine## | - | + | + | - |
| Riboflavin## | + | + | + | + |

*Carbon Sources; **Nitrogen sources; # Aminoacid sources; ## Vitamin sources
++ Prominent growth; + Moderate growth; - No growth

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