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ASSESSMENT OF ROOT-COLONIZING BACTERIA OF SOME MEDICINAL PLANTS IN LINE WITH BIOLOGICAL CONTROL

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Abstract

Microbial count densities in the rhizosphere of eleven medicinal plants were determined. The least populations were found in the rhizosphere of M. chamomilla and M. hortensis among eleven plants. The bacterial cultures were successfully isolated in pure form from the rhizosphere of the tested medicinal plants. Cultural and morphological characteristics of these isolates showed that they belong to bacilli, azotobacter, fluorescent pseudomonads and actinomycetes. These isolates were screened, in vitro, according to their capacities to produce plant growth promoting substance i.e. indol-3-acetic acid, phosphate, potassium solubilization, chitinase activities and hydrogen cyanide. The indol-3-acetic acid, gibberellic acid (GA3) and trans-zeatin riboside(t-zr) produced by the identified strains were determined by HPLC analysis. These strains proved to be effective PGPR inoculants as they possess a number of traits useful for plant growth.

Key words- root-colonizing bacteria, medicinal plants, biological control

Introduction

Medicinal plants, Antagonistic effect, Trans-zeatin riboside, 16S rRNA gene sequence. Plant growth-promoting root-colonizing bacteria (PGPR) are bacteria colonizing rhizospheres of plant that enhance plant growth through various mechanisms like nitrogen fixation, solubilization of phosphate, quorum sensing, etc. (Bhattacharya and Jha, 2012). PGPR offer various ways to replace chemical fertilizers, pesticides, etc., and thus this quality has significantly led to their increased demand. Before we start with the current applications and state of the art related to PGPR and medicinal plants, it will really be interesting to know the basic and history behind this wonderful science. Basis of application of plant

growth-promoting bacteria may be said to be led days back when Theophrastus (372–287 B.C.) suggested mixing of different soil samples to remove defects of one and add life to soil (Tisdale and Nelson 1975). Certainly the technical approach behind the same only became clear after microscopy came into play. Establishment of legumes on cultivable land was recorded for the first time by Virgil (Chew 2002). Studies have reported that application of PGPR increases nodulation and nitrogen fixation in many plants including soybean (Zhang et al. 1996). PGPR have both direct and indirect mechanisms to promote growth and yield of crop plants. Rhizosphere colonization accounts for siderophore (Schippers et al. 1988), antibiotic (Weller 1988), and hydrogen cyanide (Stutz et al. 1986) production. PGPR as Biofertilizer Biofertilizers are the substances prepared from living microorganisms which, when applied to the seeds or plant surfaces adjacent to soil, can colonize rhizosphere or the interior parts of the plants and thereby promote root growth. Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, and Sinorhizobium are reported as the potent PGPR strains for their ability to act as biofertilizers(Vessey 2003). In rhizospheric relationship, the PGPR can colonize the rhizosphere, the surface of the root, or even the superficial intercellular spaces of plant roots (McCully 2001). It is only due to the changes in different physicochemical properties of rhizospheric soil such as soil pH, water potential and partial pressure of O_2, and plant exudation as compared to the bulk soil that in turn can affect the ability of PGPR strains to colonize the rhizosphere (Griffiths et al. 1999). In endophytic relationship, PGPR reside within the apoplastic spaces inside the host plants. There is a direct evidence of existence of endophytes in the apoplastic intercellular spaces of parenchyma tissue (Dong et al. 1997) and xylem vessel (James et al. 2001). The best examples can be cited from legume–rhizobia symbioses in leguminous plants (Vessey 2003).

PGPR as Plant Growth Enhancement

Enormous PGPR are known to promote plant growth, crop yield, seed emergence, etc., thus promoting agriculture (Minorsky 2008). Plant properties like leaf area, chlorophyll content, total biomass, etc. are enhanced by inoculation of PGPR (Baset Mia et al. 2010). They also studied the effect of PGPR on external layers of root cortex of maize and wheat seedlings. Increasing demand for food and improving environmental quality have focused on the importance of PGPR in agriculture. Dobbelaere et al. (2001) assessed the inoculation effect of Azospirillum sp. on the development of agriculturally important plants and observed a noteworthy boost in the dry weight of both the root system and aerial parts of the PGPR-inoculated plants, resulting in better progress and flowering. Foliar applications of root-colonizing bacteria l

microbes in mulberry and apricot and their better development in leaf area and chlorophyll production were investigated by Esitken et al. (2003). Bacillus subtilis, B. licheniformis, Achromobacter xylosoxidans, B. pumilus, Brevibacterium halotolerans, and Pseudomonas putida are identified as having critical roles in cell elongation, escalating ACC deaminase activity, and plant growth promotion (Sgroy et al. 2009). The effect of Pseudomonas fluorescens on tomato and cucumber roots was studied by Saravanakumar and Samiyappan (2007). Seeds of various crops and ornamental plants bacterized with a mixture of PGPR and rhizobia before planting resulted in enhanced growth and disease resistance (Zehnder et al. 2001). Growth responses in wheat after the inoculation with root-colonizing bacteria basically depends on various factors like plant genotype, nature of PGPR inoculants, as well as environmental conditions as observed by Khalid et al. (2004). The root inoculation of apple tree with Bacillus M3 and Microbacterium FS01 (Karlidag et al. 2007) and the effect of arbuscular mycorrhizal(AM) fungi and PGPR in soils differing in nitrogen concentration (Ahanthem and Jha 2007) are few other important studies in this field. It was found that enhancing apple tree growth in the study might be due to enhanced production of plant growth regulators and mobilization of available nutrients by PGPR. The main objectives of this study were to isolate and characterize the root-colonizing bacteria associated in the rhizosphere of some medicinal plants, to find out high efficient bacterial isolates have the capacities to be used as plant growth promoting and bio-control agents.

Rhizosphere Microbes Interactions in Medicinal Plants

The diversity and functions of microbes in the rhizosphere, a narrow region around the root, are related to the root exudates (proteins and sugars), biogeochemical reactions and respiration (Narula et al. 2009). The rhizosphere contains abundant bacteria, fungi, protozoa and nematodes. Some nematodes are feeding on bacteria and fungi. The root exudates in the rhizosphere may control disease suppression and help in nutrient cycling. The different compounds secreted by plant roots into the rhizosphere perform multiple functions. For example, strigolactones stimulate the colonisation of the mycorrhiza fungi and germination of the parasitic plant such as Striga. The flavonoids secreted by the roots of leguminous plants increase the growth of symbiotic and non-symbiotic nitrogen-fixing bacteria, root nodules and nitrogen uptake by plants. Allelochemicals can inhibit the growth of other microorganisms in the rhizosphere, and therefore interactions are complex. In the mycorrhizosphere around the mycorrhiza-colonised roots, most of the actively absorbing rootlets are extended to the surrounding soil for nutrient uptake (Johansson et al. 2004). Since mycorrhizal fungi stimulated by some

root exudates may modify root morphology and metabolic functions, the volume of the mycorrhizosphere soil is larger than the rhizosphere soil (Linderman 1988), and root exudates in the mycorrhizosphere is quantitatively and qualitatively different from that in the rhizosphere (Leyval and Berthelin 1993; Rygiewicz and Andersen 1994) producing the 'mycorrhizosphere effect' (Linderman 1988). In addition, mycorrhizal fungi can produce antibiotics that may reduce bacterial activity in sandy soil (Olsson et al. 1996). Medicinal plants are a rich source of bioactive compounds (Toussaint et al. 2007), and these are thought to be safe to human beings and the environment compared to the synthetic medicines for the treatment of cancer and many other diseases (Nema et al. 2013). The use of medicines of plant origin has a long tradition in Europe and Asia such as traditional Chinese medicine, Indian Ayurvedic medicine and herbal medicine. More than 600 medicinal plants, comprising more than 30 % of known plant species, are recorded in the Chinese Materia Medica, citing the first use of medicinal herbals in China as early as 1100 BC(Cragg et al. 1997; Joy et al. 1998). With the increased population pressure, costs and side effects and the development of resistance to allopathic drugs for infectious diseases, the uses of medicines of plant sources for a wide variety of human ailments are increasing. So, large-scale productions of medicinal plants using modern cultivation technologies are being practised across Asian countries, to meet the demand of medicinal plants. The pests and diseases of plant are hampering the growth and quality of medicinal plants. In addition, excessive use of pesticides may degrade the quality of medicinal plant products. Therefore, the development of innovative technologies for cultivation of medicinal plants is required.

Materials and methods

Soil samples were collected from the rhizosphere of eleven medicinal plants i.e., Basil, Marrubium, Melissa, Origano, Quisqualis, Goldenrod, Melilotus, Lemon grass, German chamomile, Thyme and Margoram grown in loam sandy soil to isolate the rhizospheric bacteria.

Rhizosphere microflora of the eleven medicinal plants were counted and isolated on their selective media; spore forming bacteria and total microbial flora on nutrient agar medium, Pseudomonads on King's B medium (King et al., 1954), actinomycetes on glycerol nitrate agar medium (Waksman, 1961), azotobacters on modified Ashby's medium (Abd El-Malek and Ishac, 1968) and fungi were counted on Potato Dextrose agar medium (ATCC, 1982). Bacterial cultures were maintained on the selective medium. Bacterial colonies were selected according to the cultural and morphological characteristics including pigments; colony form, elevation and margin; texture and opacity (Simbert and Krieg, 1981).

Hydrogen cyanide production

The collected isolates were cultured in liquid media supplemented with 4.4 g/l glycine to detect HCN production according to Bakker and Schippers (1987).

Sequence analysis for PGPR bacteria

The most efficient isolates were identified using 16S rRNA gene sequence analysis. Isolation of cellular DNA was performed as described by Ausubell et al. (1987) and amplification of the 16S rRNA gene was carried out by PCR using universal primers, the forward and reverse primer (Lane, 1991). Amplification was confirmed by analysis of each PCR reaction mixture on agarose gel (1%). Sequencing of the PCR product was made on GATC German Company using ABI 3730 x1 DNA sequencer. Sequencing data were analyzed by two different computer alignment programs, DNA star (DNA STAR, Inc. USA) and sequence Navigator (Perkin, Corp., USA). Phylogenetic analysis was constructed by the neighbor – joining method based on 1000 bootstraps.

Results and discussion

In the present study, total microbial flora, actinomycetes, spore forming bacteria, fungi, fluorescent pseudomonads and azotobacters enumerated in the rhizosphere soil samples of eleven medicinal plants (viz., Ocimum basilicum, Marrubium vulgare, Melissa officinals, Origanum syriacum, Quisqualis indica, Solidago virgaurea, Melilotus officinalis, Cymbopogon citratus, Matricaria chamomilla, Thymus vulgaris and Majorana hortensis) are given in Table 1. The fluorescent pseudomonads were completely absent from the roots of M. hortensis and C. citratus. Higher densities of actinomycetes and azotobacters were reported for M. officinalis and T. vulgaris respectively, than other medicinal plants. Tamilarasi et al. (2008) reported that the varying degree of population observed in the roots of the plants is due to the effect of the chemical composition of root exudate of the individual plants on the microorganisms.

Table 1. Densities of total bacterial counts, spore forming bacteria, azotobacter, fluorescent pseudomonads, actinomycetes and fungi (cfu/g dry soil) in the rhizosphere of different medicinal plants grown in loam sandy soil

Common name of medicinal plant	Total bacterial counts($\times 10^{10}$)	Spore forming bacteria($\times 10^{7}$)	Azotobacters($\times 10^{8}$)
Basil	230.00	332.0	51.47
Marrubium	0.06	24.0	12.00
Melissa	0.24	9.60	50.57

Origano	0.04	16.00	20.0
Quisqualis	0.87	275.00	43.47
Goldenrod	0.38	77.00	20.00
Melilotus	0.80	1.38	11.53
Lemon grass	1.00	17.80	31.96

Bacterial identification

The most efficient isolates C110, Th98 and Th75 were identified based on sequence of 16S rRNA gene. Amplification of 16S rRNA gene resulted in a specific DNA fragment of approximately 1500 bp. DNA sequence analysis of the PCR products using the BLASTN program revealed that C110, Th98 and Th75, bacterial isolates had the closest similarity to Bacillus thuringiensis C110 (98% similarity), Pseudomonas fluorescens Th98 (97% similarity) and Pseudomonas poae Th75 (97% similarity). This result indicated that the most potent strains were B. thuringiensis C110, P. fluorescens Th98 and P. poae Th75. Phylogenetic trees based on partial sequences of 16S rRNA gene showed the cluster relationship of the isolates among their groups.

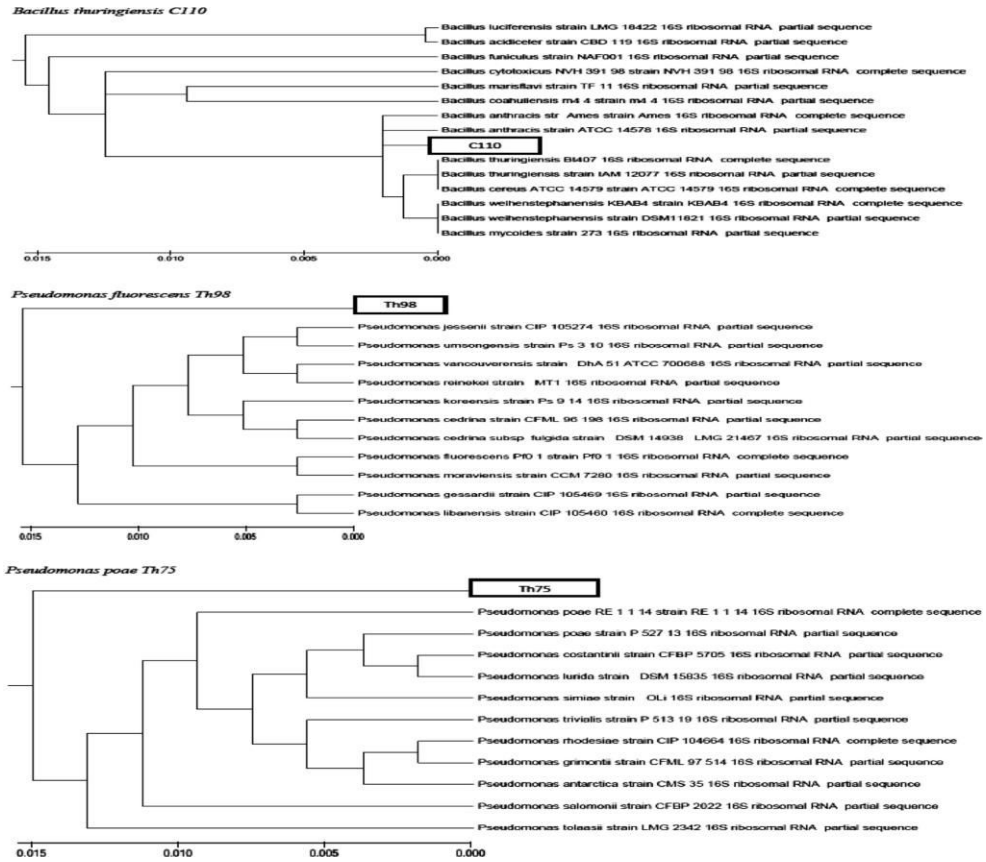

Fig. 1 Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between isolated strains C110, Th98, Th75 [shown in boxes] and related taxa. Bootstrap percentages (based on 1000 replicates) are shown if greater than 50%.

Quantification of phytohormones and soluble phosphate produced by identified strains

HPLC is a useful tool to identify and confirm the metabolites that were screened by manual method and were found to be not very reliable. All strains showed peaks comparable with the peaks at its retention time of standard indole-3-acetic acid (IAA), gibberellic acid (GA3) and trans-zeatin riboside (t-zr) on HPLC system. Results indicate that the three strains had abilities to synthesize the (IAA), (GA3) and (t-zr) in liquid culture. Maximum concentrations of these hormones were recorded by B. thuringiensis C110 whereas the lowest amount was produced by P. poae Th75. All cultures also produced soluble phosphate in variable amounts, P. poae Th75 represents the superior producer of soluble phosphate. (Silini et al., 2012) reported that phosphate solubilization by root-colonizing bacteria l isolates has been shown to be related to the production of organic acid such as formic, acetic, propionic, lactic, glycolic, fumaric and succinic acid.

Conclusion

PGPR enhance plant growth by direct and indirect means, but the specific mechanisms involved have not all been well characterized. The present review indicates the advances and formulations of PGPR in biological promotion of different characteristics of plant growth. Most PGPR isolates significantly increase plant height, root length, and dry matter production in various agricultural crops like potato, tomato, maize, wheat, etc. One of the promising approaches of replacing the use of chemical fertilizers is developing stable formulation of antagonistic PGPR in sustainable agricultural systems. Another approach is through activation of octadecanoid, shikimate, and terpenoid pathways which in turn assists the plant growth promotion. Plenty of research in this field is going on and various are fruitful too. It can be concluded that vigilantly controlled field trials of crop plants inoculated along with root-colonizing bacteria are necessary for utmost commercial exploitation of PGPR strains. This study illustrated that rhizospheric bacteria of medicinal plants have multiple PGPR traits. This led to the selection of effective isolates relying on their multiple PGPR traits and identified as B. thuringiensis C110, P. fluorescens Th98 and P. poae Th75. So, such type of study is necessary as it advocates that the use of PGPR as inoculants or biofertilizers which will be an efficient approach to replace chemical fertilizers and to enhance the growth and productivity for medicinal plants.

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