PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) FOR GROWTH IMPROVEMENT IN COCOA

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Introduction

Cocoa is a perennial tree crop traditionally and predominantly cultivated beneath coconut and arecanut palms in India. Several workers have reported presence of very high microbial biomass (Bopaiah and Shetty, 1991) nitrogen fixing *Azospirillum* (Govindan and Purushothaman, 1985) and plant benefical actinomycetes (Barreto *et al.,* 2008) in the rhizosphere of cocoa. However, not much research has gone into studies related to the plant growth promoting rhizobacteria (PGPR) of cocoa, particularly the *Bacillus* and *Pseudomonas spp*. These PGPRs have vast scope to be used as biofertilizers (Vessey, 2003). Recent research has identified isolates of many endophytic fungi, especially, *Trichoderma* species (Bae *et al.,* 2009) and bacteria, *Bacillus* spp. (Rachel *et al.,* 2008) from cocoa which have been tested for their plant growth promotion and pathogen suppression capacities.

In this study, we report our findings of the PGPR strains isolated from rhizospheric soil and roots of cocoa trees growing in different agro-ecological zones of southern India and their capacities to promote growth under green house and polybag conditions.

Materials and Methods

Isolation of soil and root-associated PGPRs

Rhizosphere soil (roots + adhering soil) was collected from cocoa (Forastero variety) growing in Kerala, Karnataka, Tamil Nadu and Andhra Pradesh for isolation of putative PGPRs. The fluorescent *Pseudomonas spp*. strains were isolated from the samples by using the serial dilution technique in King's B (KB) medium (King, 1954), S2 medium (Gould *et al.,* 1985) and *Bacillus spp*. on Nutrient agar (NA) medium. The isolates were then screened for traits that might be associated with ability to function as PGPR.

In vitro tests for plant growth promoting traits

All the isolates were screened for N-fixation, phosphate solubilization, ammonification, ACC deaminase activity, IAA, siderophore, HCN, antibiotics and chitinase production. Indole acetic acid production was determined as described by Brick *et al.,* 1991, with slight modification. ACC deaminase activity was detected in a minimal medium (Dworkin and Foster, 1958) containing 3 mM ACC as the sole source of nitrogen. Siderophore production was determined in CAS plate as described by Schwyn and Neilands (1987). Chitinase activity was tested as given by Renwick *et al.,* 1991, in a defined medium composed of colloidal chitin. Cyanide production was determined as described by Bakker and Schipper, 1987. Solubilization of phosphate was determined by growing the isolates on Pikovskaya's agar (HiMedia). Ammonification was determined by using Nessler's reagent. Growth on N-free medium was determined by using Jensen's medium (HiMedia). For detecting the production of antibiotics, supernatants of the individual strains grown in appropriate broth were transferred to wells that was made on TSA (Tryptic Soya Agar) plates and incubated for 24 hours. Presence of inhibition zone around the colonies was observed.

In vitro test for determining growth promotion (Seedling bioassay)

Cocoa isolates were tested on short duration test plant, cowpea, for determining their plant growth promoting potential. Bacterization of the sterile seeds was achieved by soaking seeds in broth, for 10 minutes. Seeds were aseptically transferred to petriplates with soft agar. Observations were taken on the 7^{th} day. Seedling bioassays were done in a growth chamber, which was maintained at $25^{0}C$, 85% humidity with 10 hrs light and 14 hrs dark cycles. Increases in seedling length and fresh weight compared to control were measured.

Green house studies

For the evaluation of growth promotion under green house conditions, seeds were sown in plastic cups filled with soil and sand mixture. The inoculation treatments were setup in a randomized design with 20 replicates/ culture. Isolates were grown in their respective medium and inoculation was carried out by soil drench with 1 ml of a bacterial suspension, which resulted in inoculum density of 106 cfu/ml. Plants were kept under natural photoperiod (16 h light/8 h dark) in the greenhouse. After 20 days of inoculation, seedlings were harvested and seedling length, fresh weight and dry weight were measured.

Evaluation of plant growth promotion ability of the selected isolates on cocoa seedlings

For evaluation of growth promotion under polybag conditions seedlings were treated with fresh suspension of respective isolates. Experiments were conducted using one month old healthy seedlings of cocoa (Bulk Forastero) obtained from CPCRI, Region Station, Vittal, Karnataka. Isolates having 108 cells ml^{-1} was used as inoculum for 1-2 months old cocoa seedlings planted in polybags containing potting mixture. A completely randomized design was employed with 20 replicates per treatment. 500 ml of respective isolates were applied as booster dose after three months of planting. At the end of the experimental period (six months), the cocoa seedlings were uprooted and growth parameters were recorded.

Results and Discussion

Isolation and *in vitro* tests for plant growth promoting traits

A total of 519 cocoa isolates were selected and purified from the media based on colony morphology and fluorescence under UV light, which include 144 rhizospheric fluorescent *Pseudomonas* spp., and 16 endophytic fluorescent *Pseudomonas* spp., 185 rhizospheric *Bacillus* spp. and 174 endophytic *Bacillus* spp. (Table 1).

All the 519 cocoa isolates were screened for N-fixation, phosphate solubilization, ammonification, ACC deaminase activity, IAA, siderophore, HCN, antibiotics and chitinase production. Rhizosphere *Bacillus* spp. showed high nitrogen fixing capacity 50.2% than endophytic *Bacillus* spp. (25.3%) whereas nitrogen fixing ability was less among fluorescent *Pseudomonas* spp. Phosphate solubilization was high among fluorescent *Pseudomonas* spp. than *Bacillus* spp. Percentage of ammonification was more than 90% except in the case of endophytic *Pseudomonas* spp. (56.3 %). Siderophore and IAA production was higher in fluorescent *Pseudomonas* spp. than *Bacillus* spp. Both rhizosphere and endophytic *Pseudomonas* spp. showed 75% IAA production. Endophytic *Bacillus* spp. (14.4%) and rhizosphere fluorescent *Pseudomonas* spp. (13.2%) were the better HCN producers than rhizosphere *Bacillus* spp. (8.6%) and endophytic fluorescent *Pseudomonas* spp. (6.3%). Most of the isolates could utilize ACC as the sole source of nitrogen. 79% of the rhizospheric fluorescent pseudomonads, 88% endophytic fluorescent *Pseudomonas* spp. 71% of rhizospheric *Bacillus* spp. and 60% of endophytic *Bacillus* spp. showed ACC deaminase activity while less number of isolates from cocoa showed chitinase activity and antibiotic production. Screening results of PGP traits are depicted in Fig. 1.

All the isolates were assessed and scored based on plant growth promoting traits and 104 isolates having combination of PGP activities (score of 9 and above) were selected for plant growth chamber assay, for testing their PGP efficacy under *in vitro* conditions. All the traits of PGPR might not get expressed at a given point of time. Thus, more the presence of the PGPR traits in an individual organism, more the chances of the organism being a successful inoculant strain. Similar multiple PGP activities among PGPR have been reported earlier (Ahmad *et al.,* 2008).

In vitro test for determining growth promotion (Seedling bioassay)

Percentage increment in different PGP parameters, studied in growth chamber was higher in treatments using *Pseudomonas* sp. KDSF 7 (100%) and *Bacillus* sp. TSB 15 (57%). 58 isolates induced total seedling length as compared to non-treated control. 88 isolates which increased any one of the parameters like shoot length, root length or fresh weight of cow pea seedlings in growth chamber assay were selected for greenhouse studies.

Green house studies

Thirty-three cocoa isolates showed increase in all the three parameters *i.e.,* seedling length (SL), fresh weight (FW) and dry weight (DW). This includes 7 fluorescent *Pseudomonas* spp., 16 endophytic *Bacillus* spp. and 10 rhizospheric *Bacillus* spp. Among *Bacillus* spp. WEB 6 showed maximum increase in growth parameters (13%, 38% and 36% respectively in SL, FW and DW). An incremental effect on growth observed in cowpea on inoculation with rhizobacteria indicated a broad spectrum plant growth promoting activity by the isolates.

Evaluation of plant growth promotion ability of the selected isolates on cocoa seedlings

Based on the three screening studies (*in vitro* characterization, seedling bioassay and green house studies) 21 isolates were selected and tested for their plant growth promoting effect on cocoa seedlings under polybags conditions. All isolates selected for plant growth experiments, significantly increased at least one aspect of cocoa seedling growth. Statistically significant increase (P= 0.05) over the control was observed in the growth parameters such as total seedling length, fresh weight and dry weight of shoot and root and collar girth of cocoa seedlings when they were inoculated with PGPR cultures. The total dry mass across treatments ranged from 2 to 58%. A maximum increase of 41% collar girth was recorded in cocoa seedlings inoculated with fluorescent *Pseudomonas* sp. KDSF 23, *Bacillus* sp. ASB3, *Bacillus* sp. VEB 4 and Bacillus sp. KGEB 16 were found to be the best plant growth promoters for cocoa seedlings.

Table 1. Details of putative PGPR isolates obtained from soil and roots of cocoa

State	Name of place	Total number of isolates obtained			
		Rhizosphere		Roots	
		Bacillus spp.	Fluorescent *Pseudomonas* spp.	*Bacillus* spp.	Fluorescent *Pseudomonas* spp.
Kerala	Kasaragod	29	12	13	28
	Wayanad	20	18	45	17
	Kozhikode	09	03	04	00
Karnataka	Tumkur	46	17	17	17
	Kidu	25	16	27	24
	Vittal	20	0	02	01
Tamil Nadu	Coimbatore	21	12	03	06
	Pollachi	12	05	01	03
Andhra Pradesh	Ambajipetta	18	11	15	02
		185	**144**	**174**	**16**
			Total		**519**

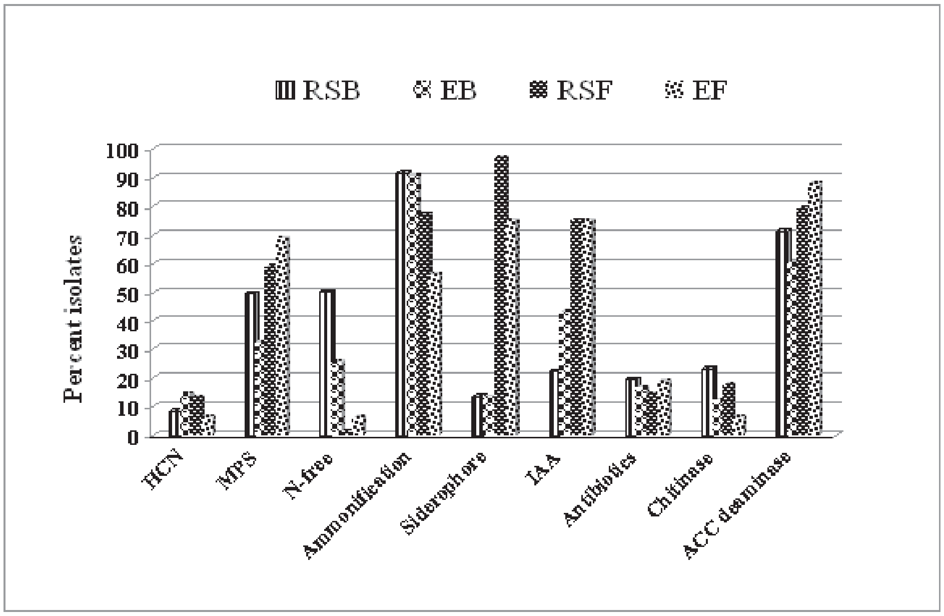

Fig. 1. Percentage of PGPR isolates showing plant growth promoting traits

RSB- rhizosphere soil *Bacillus* spp., EB- endophytic *Bacillus* spp., RSF- rhizosphere soil fluorescent *Pseudomonas* spp., EF- endophytic fluorescent *Pseudomonas* spp., HCN-production of hydrogen cyanide, MPS-mineral phosphate solubilization, N-free- growth on N-free agar medium, IAA- indole acetic acid, ACC- amino cyclopropane-1- carboxylate (ACC) deaminase.

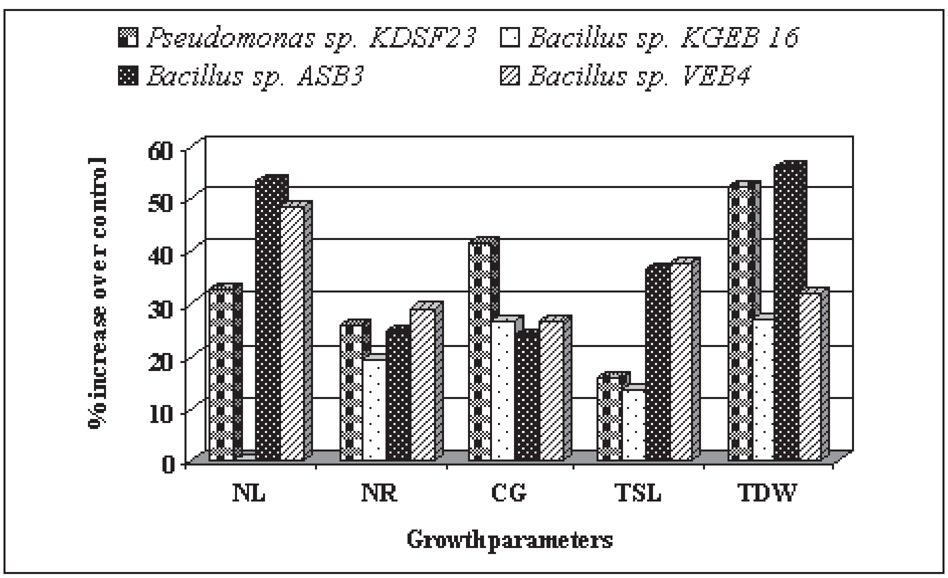

Fig. 2. Effect of inoculation of potent PGPRs on cocoa seedlings under polybag conditions

NL- number of leaves, NR- number of roots, CG-collar girth, TSL- total seedling length, TDW- total dry weight

Conclusion

The results suggest that inoculation with PGPR's had a beneficial effect on cocoa seedlings, therefore they can be utilized as inoculants for biofertilizer production for sustainable agricultural systems.

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