PLANT GROWTH PROMOTION BY RHIZOBACTERIA

for Sustainable Agriculture

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Bio-active Secondary Metabolites from PGPR and Botanicals

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Abstract

ICRISAT has a large collection of bacteria, fungi and actinomycetes with agriculturally beneficial traits isolated from various sources of composts and rhizosphere soil samples from sorghum, rice and pigeonpea crops, in addition to potent botanicals. At least 1500 accessions of plant growth promoting microorganism (PGPM viz. 89 phosphate solublizers, 252 siderophore producers, 198 cellulose degraders, 490 nitrogen fixers, 350 antagonists, 101 entomopathogens and 20 fluorescent Pseudomonads) have been isolated from the above sources in addition to 17 botanicals capable of managing *Helicoverpa armigera* and *Spodoptera litura*, the two most devastating insect pests of many crops. Bio-active secondary metabolites of the potent PGPM and botanicals (particularly on Anona, Datura, Pongamia, Parthinium, Gliricidia, Neem and Jatropha) responsible for managing *H. armigera* and *S. litura*) and antagonistic to five disease causing fungi (*Fusarium oxysporum* f. Sp. *ciceri*, *F. udum*, *F. solani*, *Sclerotium rolfsi* and *Macrophomina phaseolina*) were studied. Secondary metabolites of the potent PGPM strains and botanicals were purified by solvent partitioning, solid phase extraction, TLC and reversed-phase open column chromatography. Stages in purification were monitored by a live/dead assay employing neonates of H. armigera and S. litura or plant pathogenic fungi. Final purification will be done in HPLC and the purified active compound(s) will be identified by mass spectrometry and nuclear magnetic resonance studies. Purification of the secondary metabolites from the above PGPM and botanicals are on and results will be discussed in presentation.

Introduction

Over reliance of chemical pesticides and fertilizers has resulted in a few problems including safety risks, outbreaks of secondary pests normally held in check by natural enemies, environmental contamination, and decrease in biodiversity and insecticide resistance (Lacey and Shapiro-Ilan, 2008). Increasing cost and negative effects of pesticides and fertilizers necessitates the idea of biological options of crop protection and

production. Various biological options such as entomopathogens, antagonistic microbes, endophytes, animal wastes, botanicals and crop residues serves as an alternative to chemical pesticides and fertilizers (Rupela *et al.*, 2005). Incorporation of crop residues and application of PGPM microbial inoculants can have a direct impact not only on soil health and crop productivity but also can be an alternative for the chemical fertilizers and pesticides (Hameeda *et al.*, 2006). Majority of PGPM are isolated from rhizosphere (Khalid *et al.*, 2004). PGPM stimulate growth directly by nitrogen fixation (Han *et al.*, 2005), solublization of nutrients (Rodriguez and Fraga, 1999), production of growth hormones, 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Correa *et al.*, 2004) and indirectly by antagonizing pathogenic fungi by production of siderophores, chitinase, ß-1, 3-glucanase, antibiotics, fluorescent pigments and cyanide (Pal *et al.*, 2001). Hence it was decided to isolate PGPM from various sources of herbal compost and rhizosphere of many crops.

Microbial collection at ICRISAT has isolated and identified a large collection of bacteria, fungi and actinomycetes with agriculturally beneficial traits from various herbal composts and rhizosphere soil samples of sorghum, pigeonpea and rice. These accessions possess at least one of seven agriculturally beneficial traits studied viz. phosphate solublization, siderophore production, cellulose degradation, nitrogen fixation, antagonistic to disease causing fungi (viz. *F. oxysporum* f. Sp. *ciceri*, *F. udum, F. solani, S. rolfsi* and *M. phaseolina*), entomopathogens (against *H. armigera* and *S. litura*, the two devastating insect pests of various crops) and Pseudomonas fluorescens. Here, at ICRISAT, we are now working on bio-active secondary metabolites of the above potent PGPM and botanicals responsible for managing insect pests and disease causing fungi.

Materials and Methods

Sources of isolation of microbial cultures: Predominant microbial cultures were isolated from different natural niches, which were known for microbial diversity, namely termite soil, composts of different herbs, different flowers (dandleon, yarrow, chamomile, Billbergia spp), cow dung, garden waste, kitchen waste, surfaces of four macro-fauna viz., earthworm, slugs, mollusk and centipede which were normally present in the compost and rhizosphere soil of sorghum and rice.

Isolation of plant growth promoting microbes from the natural niche

Potential plant growth promoting microorganisms were isolated by using seven different media. Appropriate dilutions were plated on Luria Agar (PDA) for bacteria, ¼ PDA + streptomycin (500 mg L-1) for fungi, Kings, B medium (King *et al.*, 1954) for *Pseudomonas fluorescens*,

chromazurol-S(iron-free) agar medium for siderophore producers (Schwynn and Neilands, 1987), Pikovskaya's (Pikovskaya, 1948) medium for P-solubilizers (Benomyl (100mg L-1) was added to suppress fungi). Jenson's N2 free media for N2 fixers (Jensen, H.L., 1951). The plates were incubated at 28±2°C for 24-72 hrs. Colonies with desired traits on the different media were picked up and preserved for further studies.

Screening of plant growth promoting microbes against disease causing fungi

For in-vitro screening, standard dual culture test was performed, in which, the potential antagonistic isolates were grown with the pathogenic fungus. 25 ml sterilized ¼ PDA was poured in 100 mm Petri plates. The fungal discs of 6 mm2 were cut from 3 days (*M. phaseolina* and *S. rolfsii*) or 5 days (*F. solani* and *F. udum, F. oxysporum* f.sp. *ciceri*) old cultures (grown on ¼ PDA plates) and placed in the center of a ¼ PDA plate. At a distance of 3 cm around the fungus 4 different antagonistic isolates were streaked where as in the control plate, only fungal disc was kept in the centre of the plates. The plated were kept for incubation at 28°C ± 2°C till fungus fully grown in the control plate.

Separation of bioactive metabolites from herbal compost wash and PGPM cultures

Bio-washes from herbal compost namely Anona (*Anona fistulosa*), Datura (*Datura metal*), Pongamia (*Pongamia pinnata*), Parthinium (*Parthenium hysterophorus*), Gliricidia (*Gliricdia sepium*), Neem (*Azadirachta indica*) and Jatropha (*Jatropha curcas*) were collected in the sterilized bottles. The bio-washes were filtered through Whatman filter paper No 1 (to remove the debris and solid particles) and centrifuged at 10,000g for 20 min at 4°C followed by the collection of supernatants, which was used for purification of active compounds. For partial separation of the bio-active compounds present in the herbal bio-wash C18 SPE cartridge or solvent partitioning was used.

SPE protocol

SPE cartridges were solvated with 20 ml of 100% methanol and equilibrated with 20ml of 5% methanol in ultra pure distilled water. Bio-wash samples were added with methanol (to make a final concentration of 5% methanol) and slowly injected into the SPE cartridge. Non-adsorbed fraction was collected separately. The cartridge was eluted with 5 ml of 100% methanol (2ml at a time) and collected as adsorbed fraction. Both adsorbed as well as non-adsorbed fractions were checked for their activity against 5 plant pathogenic fungi as well as *H. armigera* and *S. litura*. In case of PGPM microbes, the cultures were centrifuged at 10,000g for 20min at 4°C and the supernatants were used for further purification on SPE cartridge, as per the protocol described above.

Solvent partitioning protocol: Supernatants of bio-wash or PGPM cultures (collected as per the protocol described above), were collected and adjusted for pH 3 with 0.1N H_2SO_4. These were partitioned three times against ethyl acetate. The organic and aqueous phases were separated and evaporated. The residues were collected in small volumes of methanol and checked for their activity against 5 plant pathogenic fungi as well as *H. armigera* and *S. litura*.

Evaluation of crude extracts of botanicals and plant growth promoting microbes on insect pests and plant pathogenic fungi

1. Evaluation on insect pests: Partially separated bio-active compounds from PGPM or bio-wash of botanical extract were added (50μl) in to the camphor bottle (4cm Dia. X 4.5cm ht.) which contained sterilized artificial diet (2g). In each bottle, 4 days old single larva was released. Each bottle serves as one replication. There were 25 replications for each treatment. In the control sterile distilled water was sprayed. Observations were taken on number of live larvae and their weight after 96 hrs.
2. Evaluation on plant pathogenic fungi: Partially separated bio-active compounds from PGPM microbial cultures or bio-wash of botanical extract were added (at different concentration as treatments) in to a 100ml conical flask which contained sterilized media (30ml) required for the particular plant pathogenic fungi. Control flasks contained no bio-active compounds. Flasks were inoculated with any one of plant pathogenic fungi and incubated at 26°C in a shaker for 5 days. At the end of incubation counts were done by MPN method and compared with control.

Results

At least 1500 accessions of PGPM such as phosphate solublizers, siderophore producers, cellulose degraders, nitrogen fixers, antagonists (against five plant pathogenic fungi viz. *F. oxysporum* f. Sp. *ciceri, F. udum, F. solani, S. rolfsi* and *M. phaseolina*), entomopathogens (against *H. armigera* and *S. litura*) and fluorescent Pseudomonads have been isolated from herbal composts and rhizosphere soil samples of sorghum, rice and pigeonpea crops (Table 1). ICRISAT has also identified 17 botanicals capable of managing *H. armigera* and *S. litura*, the two most devastating insect pests of many crops that includes, cotton, pigeonpea, chickpea and tomato (Table 2). Some of the botanicals such as Anona, Datura, Pongamia, Parthinium, Gliricidia, Neem and Jatropha have been found to do well against *H. armigera* and *S. litura* and hence was decided to separate the bio-active compounds (secondary metabolites) responsible for the action. Bio-efficacy of 3 different fractions (fresh crude bio-wash, C18 SPE adsorbed and non-adsorbed fraction) of the above 7 herbal bio-

wash samples were studied (Table 3). When, fresh crude bio-wash was treated with healthy *H. armigera* larvae, mortality rate varied between 31% and 38% for any given botanicals compared to un-inoculated control. However, the weight reduction over control was great in Anona and Neem (60% over un-inoculated control) followed by Pongamia (58%), Datura (56%), Jatropha (53%), Gliricidia (47%) and the least was found in Parthinium (25%). C18 SPE Adsorbed fraction killed the larvae more efficiently than fresh crude bio-wash (as found clearly in Jatropha (82%), Parthenium (73%), Neem (60%), Anona (60%), Gliricidia (58%), Pongamia (53%) and Datura (42%)) as well as weight reduction over control has been reported (Table 3). Except Datura, all other bio-washes had done exceptionally well and the difference was statistically significant at 0.001%. In the case of non-adsorbed fraction, the mortality was ranged between 13% (Neem) to 53% (Pongamia) whereas all the seven bio-washes had reduced the weight of the larvae compare to control (maximum and minimum weight reductions were observed in Datura (75%) and Neem (69.0), respectively) (Table 3). The effect of fresh crude bio-wash and C18 SPE adsorbed as well as non-adsorbed fractions on 5 plant pathogenic fungi is going. These results as well as further purification of the active fractions will be discussed in the presentation.

Table 1. Number of microbial isolates having agriculturally important beneficial traits.

Agriculturally important beneficial traits	Number of microbial isolates
Antagonists	350 (51 against FOC and 262 against *M. phaseolina* are imp.)
Entomopathogens	101
Phosphate solublizers	89
Nitrogen fixers	490
Cellulose degraders	198
Siderophore producers	252
Fluorescent Pseudomonads	20

Table 2. Evaluation of one percent botanical extract against neonates of *Helicoverpa armigera* and *Spodoptera litura.*

Botanical	Scientific names	% Mortality		% Repellency (% reduction in egg laying over control)	
		HA	SL	HA	SL
Anona	*Anona squamosa*	20	40	47	96
Anona rind	*Anona squamosa*	8	46	79	90
Anona seed	*Anona squamosa*	25	34	45	93
Calotropis	*Calotropis gagantea*	18	12	33	66
Chrysanthemum	*Chrysanthemum domestica*	38	32	65	55
Datura	*Datura metal*	40	46	32	46

Jatropha cake	*Jatropha curcas*	24	26	8	95
Marigold	*Tagetus erecta*	35	34	66	96
Melia	*Melia azedarach*	22	30	93	87
Neem	*Azadirachta indica*	42	48	9	73
Neem Fruit	*Azadirachta indica*	21	74	84	100
Parthenium	*Parthenium hysterophorus*	10	26	13	75
Pongamia	*Pongamia pinnata*	30	64	92	56
Pongamia seed	*Pongamia pinnata*	29	32	4	77
Prosopis	*Prosopis juliflora*	16	44	34	37
Rain tree	*Samanea saman*	31	44	43	ND
Rain tree Pod	*Samanea saman*	39	52	21	73
Trydax	*Tridax procumbens*	32	34	32	85
Vitex	*Vitex negundo*	10	44	1	93

ND= Not done; HA- *Helicoverpa armigera*; SL- *Spodoptera litura*

Table 3. Bio-efficacy of undiluted, adsorbed and non-adsorbed fractions of bio-washes on *H. armigera* larvae.

Bio-wash	Fresh crude bio-wash		Adsorbed Fraction		Non-adsorbed Fraction	
	Mortality (%)	Weight reduction over control (%)	Mortality (%)	Weight reduction over control (%)	Mortality (%)	Weight reduction over control (%)
Anona	38.23 (0.448)	60.33 (0.658)	59.90 (0.657)	65.00 (0.744)	33.35 (0.355)	74 (0.906)
Datura	30.70 (0.335)	56.00 (0.595)	42.36 (0.451)	71.33 (0.859)	36.7 (0.382)	75.5 (0.924)
Pongamia	37.80 (0.421)	58.33 (0.633)	53.22 (0.599)	73.00 (0.896)	53.35 (0.571)	70.5 (0.856)
Parthinium	36.03 (0.385)	24.67 (0.259)	73.33 (0.953)	89.00 (1.196)	43.35 (0.449)	73 (0.852)
Gliricidia	37.37 (0.404)	46.67 (0.493)	57.91 (0.625)	69.67 (0.829)	43.35 (0.466)	75 (0.909)
Neem	36.47 (0.399)	59.67 (0.643)	62.22 (0.702)	84.33 (1.055)	13.35 (0.134)	69.5 (0.779)
Jatropha	36.03 (0.426)	53.00 (0.562)	82.33 (1.077)	97.00 (1.373)	26.7 (0.270)	73.5 (0.875)
Control	15.57 (0.160)	NA	22.23 (0.237)	NA	3.35 (0.033)	NA
SEm	0.0715NS	0.1326NS	0.0864***	0.1828NS	0.1105NS	0.0747NS

Data in the parenthesis are arcsine transformed values; ***= Statistically significant at 0.001% NS= Statistically non significant; NA=Not applicable

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