Acta Phytopathologica et Entomologica Hungarica 39 (1–3), pp. 29–38 (2004)

Production of Secondary Metabolites by Strains of *Pseudomonas* spp. Antagonistic to *Colletotrichum falcatum* Causing Red Rot Disease in Sugarcane

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Production of secondary metabolites such as siderophores, indole acetic acid, salicylic acid and antibiotics phenazine, pyocyanine and 2,4-diacetyl phloroglucinol by *Pseudomonas* spp. associated with induced systemic resistance (ISR) activity in sugarcane against red rot disease was assessed. Many of the strains have been found to produce these metabolites and antibiotics in the culture medium. The antibiotics were separated and identified by thin layer chromatography by running the purified metabolites from the reference strains. The bacterial strains varied in their capability to produce the metabolites. The purified compounds were tested for their antifungal activity against the red rot pathogen *Colletotrichum falcatum.* Most of the purified metabolites have completely arrested conidial germination and mycelial growth of the fungus. The results suggest that the metabolites production may play a role in antagonism/induced systemic resistance against the pathogen.

Keywords: *Pseudomonas* strains, secondary metabolites, *C. falcatum.*

Fluorescent pseudomonads belonging to plant growth promoting rhizobacteria (PGPR) when introduced on seed or planting material control plant diseases caused by soil borne pathogens or promote plant growth by suppressing deleterious rhizosphere microorganisms (Weller, 1988). These soil bacteria, in particular the *Pseudomonas* spp. produce various low molecular weight metabolites, some of which are potent antifungal agents. These antagonistic bacteria suppress the pathogens by producing one or more of a variety of metabolites that include antibiotics (Thomashow and Weller, 1988, 1990; Rosales et al., 1995), siderophores, indole acetic acid (IAA) and salicylic acid. Thomashow and Weller (1988) and Pierson and Thomashow (1992) have demonstrated that production of secondary metabolites phenazine-1-carboxylate (PCA) by the *P. fluorescens* 2–79 and 30–94 strains in suppression of take-all disease in wheat caused by *Gaeumannomyces graminis* var *tritici.* Similarly, Harrison et al. (1993) have established that production of 2,4-diacetylphloroglucinol by *P. fluorescens* strain Q2–87 was essential for the suppression of *G. graminis* var *tritici* in wheat. Another antibiotic from *P. cepacia,* pyrrolnitrin was found to have strong antifungal properties against *Rhizoctonia solani.* Rosales et al. (1995) isolated several antibiotic compounds viz., PCA, pyocyanine, phloroglucinol, pyrrolnitrin, pyoluteorin and oxychloraphine from the rice associated *Pseudomonas* antagonists. These compounds have showed inhibition against the sheath blight pathogen, *R. solani.*

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These bacteria also induce systemic resistance in crop plants against variety of plant pathogens, some insect pests and nematodes (Ramamoorthy et al., 2001). The improvement and eventual commercialization of fluorescent pseudomonads as biological control agents depend in part on understanding and exploiting the mechanisms involved in these antagonistic interaction among the bacteria, pathogens and their plant hosts. The PGPR strains colonizing sugarcane rhizosphere and stalks (endophytes) were found to induce systemic resistance against *Colletotrichum falcatum* Went causing red rot in sugarcane (Viswanathan and Samiyappan, 1999a, 2000). Further studies were conducted on the production of secondary metabolites, antibiotics in culture medium and antifungal activity of the purified metabolites against *C. falcatum.*

Materials and Methods

Pathogen and Pseudomonas strains

The pathogen was isolated from the naturally infected (internodal tissue) sugarcane cv CoC 671 and maintained on oatmeal agar (OMA) medium. Single conidial culture of the fungus was obtained and maintained on the same medium. Native sugarcane rhizosphere and endophytic strains were isolated from samples obtained from different parts of Tamil Nadu state as reported earlier (Viswanathan and Samiyappan, 1999a). In addition to the strains isolated from sugarcane, the fluorescent pseudomonad strains obtained from the *Pseudomonas* culture collection of Lab#20, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore were also used for comparative study in certain tests. The list of strains used in the study is presented in *Table 1*. The reference *Pseudomonas* spp. cultures of Pf-5, Pf 2–79, Pf 30–84 R9 and PAOI were obtained from Linda S. Thomashow (USDA-ARS, Root Disease and Biocontrol Unit, Pullman, USA).

Siderophore production by the PGPR strains

Siderophore synthesis of the selected strains was determined as per the method of Reeves et al. (1983). The bacterial strains were grown in the King's medium B broth for 3 days. Supernatant from the cultures was collected after centrifugation (2000 rpm, 10 min). The pH was adjusted to 2.0 with diluted HCl before extracting with ethyl acetate. Five ml of Hathway's reagent were added to 5 ml of the ethyl acetate fraction in 50% ethanol, shaken thoroughly and absorbance was measured at 700 nm for dihydroxy phenols. The quantity of siderophore produced was expressed as µmoles of benzoic acid/ml.

Indole acetic acid production

The PGPR strains were grown in tripticase soya broth with tryptophan (100 mg/ml) for 30 h. IAA production was estimated using cell free culture filtrate with Salkowsky reagent. The absorbance was read at 530 nm and presence of IAA in the culture filtrate was quantified as mg/ml (Gorden and Paleg, 1957).

Table 1

Pseudomonas strains used in the study

Sl No	Strain	Place of collection	Host plant	Major characters
1	ARR1	Pondicherry	Sugarcane	Fluorescent
2	ARR1G	Pondicherry	Sugarcane	Fluorescent
3	ARR2	Pondicherry	Sugarcane	Fluorescent
4	CHAO	Switzerland	Tobacco	Non fluorescent
5	EP1	Nellikuppam	Sugarcane	Fluorescent
6	FP7	Trivandrum	Rice	Fluorescent
7	KKM1	Kumbakonam	Sugarcane	Fluorescent
8	Pf1	Vamban	Urdbean	Fluorescent
9	PfAR1	Aliyarnagar	Groundnut	Fluorescent
10	PfATR2	Attur	Tapioca	Fluorescent
11	PfKO2	Kodaikanal	Pepper	Non fluorescent
12	PfNA4	Namakkal	Banana	Fluorescent
13	PFNL1	Nilgris	Forest	Non fluorescent
14	PfRA1	Rajapalayam	Carrot	Fluorescent
15	PfSA1	Sattur	Maize	Fluorescent
16	VPT10	Coimbatore	Sugarcane	Fluorescent
17	VPT4	Coimbatore	Sugarcane	Fluorescent
18	Pf-58*			
19	Pf 2–79*			
20	PAOI*			
21	Pf 30–84 R9*			

* Reference strains.

Salicylic acid (SA) production

The PGPR strains were grown in the standard succinate medium for 48 h at 27 ± 2 °C (Meyer and Abdallah, 1978). The culture supernatants were acidified to pH 2.0 with 1N HCl and SA was extracted in chloroform. The absorbance of the purple iron-SA complex, which developed in the aqueous phase, was measured at 527 nm. The quantity of SA was expressed as µg SA/ml of culture filtrate (Meyer et al., 1992).

Antibiotics production by the PGPR strains

For the extraction of secondary metabolites the different strains were cultivated on the pigment production medium (PPM) containing peptone 20 g; glycerol 20 g; NaCl 5 g; KNO_3 1 g; distilled water 1000 ml (pH 7.2). The PGPR strains and reference strains were cultured on PPM broth and incubated in a rotary shaker for 4 days at room temperature (150 rpm; 28 ± 2 °C).

Extraction and isolation of phenazine, pyocyanine and oxychlorophine

The antibiotics pyocyanine, phenazine-1-carboxylic acid (PCA) and oxychlorophine were separated as described by Rosales et al. (1995). The three pigments PCA, oxychlo-

rophine and pyocyanine were recovered from $NaHCO_3$, benzene and chloroform fractions from the PPM broth cultures, respectively. The antibiotic metabolites were air dried and dissolved in 1 ml of methanol.

Extraction of 2,4-diacetylphloroglucinol

The cultures were grown for 4 days in PPM broth on a rotary shaker at 30 °C. The supernatant was collected after centrifugation (3000 rpm; 5 min) and acidified to pH 2.0 with 1N HCl and then extracted with equal volume of ethyl acetate. The extracts were reduced to dryness *in vacuo* and the residue was dissolved in 100 µl of methanol.

Thin layer chromatography

The metabolites were purified by thin layer chromatography (TLC) on 250 µm thick silica gel. Isopropanol/ammonia/water (8:1:1) solvent system was used for the purification of PCA, oxychlorophine and pyocyanine. Acetonitrile/methanol/water (1:1:1) solvent system was used for other metabolites. Ten µl of respective metabolites in methanol were spotted on the gel plates before running. After running, the plates were viewed under UV light at 254 nm to locate the spots and plates were sprayed with diazotized sulfanilic acid (DSA) (Rosales et al., 1995). The spots of the individual strains were marked and Rf values of the metabolites from the reference strains *P. fluorescens* 2–79 (phenazine), *P. fluorescens* CHAO and Pf-5 (phloroglucinol), *P. fluorescens* Pf 30–84 R9 (phenazine) and *P. aeruginosa* PAIO (pyocyanine) were compared.

Antifungal effects of the metabolites

The marked spots in the TLC plates were scrapped and dissolved in 100 µl methanol, centrifuged to separate silica. The eluant was assayed against *C. falcatum* conidial germination and mycelial growth. About 20 µl of eluant was allowed to dry in a cavity slide and later 20 µl of *C. falcatum* conidial suspension (10^6 conidia/ml) was placed and kept in a moist chamber for 24 h to record conidial germination. For recording conidial germination, the slides were viewed in a binocular microscope (200×). From each microscopic field about 20 conidia were counted and in each cavity ten such observations were made. For each treatment at least five such observations were made and results were expressed as per cent germination. To assess their efficacy against mycelial growth, 20 µl of eluants were placed in sterile microtitre plate wells containing 100 µl of solidified oat meal agar. The methanol was allowed to dry in the laminar flow chamber for 60 min and later 20 µl of the conidial suspension was placed in the wells and incubated at room temperature (28 ±2 °C) in sterile condition. The mycelial growth was observed after 96 h.

Results

Assessment of siderophore production in the culture medium showed that among the 17 PGPR strains PfAR1, PfNL1, FP7, Pf1, VPT4, VPT10, PfKO2, PfRA1 and CHAO have

produced more amount of siderophore in the culture medium. The strains ARR1 and FP7 have recorded very high amounts of IAA in the culture medium as compared to the other strains. EP1, PfATR2, PfKO2, PfAR1, PfRA1 and VPT4 were the other strains which produced high amounts of IAA. Variation in SA production by different strains of PGPR in the succinate medium was observed. Among the different strains CHAO, ARR1O, ARR1G, Pf1 and PfAR1 have recorded higher SA in the medium *(Table 2).*

Table 2

Production of different secondary metabolites by *Pseudomonas* spp. associated with sugarcane and other crops

Bacterial strains	Secondary metabolites					
	Pyocyanine	Phenazine	Phloroglucinol	Siderophore[1]	IAA[2]	Salicylic acid[2]
ARR1	+ (0.58)	+ (0.65)	–	NT	185.60i	NT
ARR1G	+ (0.58)	–	–	6.94d	20.44a	21.53f
ARR1O	NT	NT	NT	6.26e	NT	30.51g
ARR2	+ (0.58)	–	+ (0.95)	4.97gh	NT	13.57cde
EP1	+ (0.58)	–	+ (0.93)	6.40e	54.55ef	1.17a
FP7	–	–	+ (0.93)	10.94b	160.14h	21.02f
KKM1	–	–	+ (0.93)	7.34d	38.14bc	17.83ef
MDI1	NT	NT	NT	6.04ef	NT	NT
Pf1	–	–	+ (0.78)	8.51c	49.57de	22.53f
PfAL1	NT	NT	NT	NT	NT	8.89bcd
PfAR1	NT	NT	NT	16.38a	62.72fg	20.94f
PfATR2	NT	NT	NT	4.17h	54.57ef	12.61cde
PfKO2	NT	NT	NT	8.51c	71.10g	2.79ab
PfNL1	+ (0.58)	+ (0.56)	–	10.01b	NT	NT
PfNA4	NT	NT	NT	NT	NT	7.84abc
PfRA1	NT	NT	NT	8.61c	50.58e	15.26def
PfSA1	NT	NT	NT	5.33fg	40.00cd	NT
VPT4	–	+ (0.50)	+ (0.83)	8.31c	53.87ef	16.24ef
VPT10	–	–	+ (0.86)	8.67c	28.49ab	1.08a
CHAO*	–	+ (0.58)	+ (0.84)	8.61c	35.17bc	35.48g
Pf 5*	–	–	+ (0.78)	NT	NT	NT
Pf 2–79*	+ (0.58)	+ (0.62)	–	NT	NT	NT
PAOI*	+ (0.58)	+ (0.50)	+ (0.83)	NT	NT	NT
Pf 30–84 R9*	–	+ (0.65)	–	NT	NT	NT

NT: Not tested; *: standard metabolite producing reference strains; –: non production of antibiotics in the medium; +: production of antibiotics in the medium, values in the parentheses indicate the Rf values of the metabolites; 1: values are µmoles benzoic acid/ml of culture filtrate; 2: µg/ml culture medium. In a column values followed by the same letters are not significant (P = 0.05%) by DMRT.

The reference strains and PGPR strains native to sugarcane were grown in the pigment production medium and the antibiotics were purified using different solvents. The results indicated that most of the strains produced pyocyanine at a higher level except CHAO, FP7 and VPT4. However, the strains CHAO and VPT4 recorded higher produc-

tion of phenazine. The strains ARR2, EP1, FP7, KKM1, Pf1, VPT4 and VPT10 produced phroroglucinol in the culture medium. The individual metabolites were separated by thin layer chromatography. The results showed that the strains ARR1, ARR1G, ARR2, EP1 and PfNL1 were positive for the pyocyanine, the strains ARR1, PfNL1 and VPT4 were positive for phenazine and the strains ARR2, EP1, FP7, KKM1, Pf1, VPT4 and VPT10 were positive for phloroglucinol *(Table 2).*

The metabolite fractions were eluted from the TLC plates after colour development and assessed for their effect on *C. falcatum* conidial germination. The results showed that pyocyanine from all the strains including the reference strains were inhibitory to the fungal conidial germination. Phenazines of all strains were highly antifungal and here it was found that reference strains had comparatively lesser effect on the fungus than the test strains. Phloroglucinol from the strains FP7 and KKM1 has inhibited the conidial germination completely *(Table 3).* In microtitre plate assay, it was found that the purified metabolites arrested mycelial growth of the pathogen completely. By 96 hrs in the

Table 3

Effect of *Pseudomonas* metabolites on *C. falcatum* conidial germination

Pseudomonas strain	Per cent inhibition to conidial germination		
	Phenazine	Pyocyanine	Phloroglucinol
ARR1	98.08 (84.27)b	100.00 (89.65)	–
ARR1G	–	97.44 (84.08)	–
ARR2	–	100.00 (89.65)	89.21 (78.44)abc
CHAO	96.58 (79.68)b	–	85.34 (73.05)a
EP1	94.69 (80.39)b	–	–
FP7	–	–	100.00 (89.73)c
KKM1	–	–	100.00 (89.73)c
Pf1	90.60 (77.03)b	–	94.93 (80.91)abc
PfNL1	96.05 (82.72)b	97.42 (84.40)	–
VPT4	96.31 (82.27)b	–	96.84 (84.14)abc
VPT10	–	–	91.38 (77.70)ab
Pf5	–	97.17 (84.62)	97.48 (85.92)bc
Pf 2–79	93.32 (79.50)b	100.00 (89.65)	88.29 (74.67)ab
PAOI	80.92 (66.88)a	98.33 (85.68)	95.78 (82.55)abc
Pf 30–84 R9	89.55 (74.27)ab	–	94.84 (82.05)abc

– Not tested.
In a column means followed by the same letters are not significant at 5% level by DMRT.
Values in the parentheses are arc sine transformed values.

untreated plate wells, greyish cottony mycelial growth covering entire well surface was noticed whereas, in the antibiotics treated plate wells no such mycelial growth was noticed. The results indicated that purified metabolites inhibited fungal growth at tested concentrations.

Discussion

The PGPR strains induce physiological changes in entire plants, making them more resistant to pathogens. This phenomenon, termed as induced systemic resistance (ISR), has been demonstrated for various rhizobacteria in several plants (De Meyer et al., 1999; Wei et al., 1991; Van Peer et al., 1991). The induced resistance reduces disease symptom of a wide range of pathogens and the bacterial strains bring about changes in the host through different mechanisms (Van Loon et al., 1998; Ramamoorthy et al., 2001). Fluorescent pseudomonad strains produce siderophores such as pyoverdine, pyochelin and SA and they were implicated in biocontrol mechanisms for many pathogens (Weller, 1988). Fluorescent siderophores are unique to pseudomonads – a trait that has implicated these organisms as PGPR. Fluorescent siderophores have been isolated from soil and there is considerable genetic and biochemical evidence that demonstrates their role in the promotion of plant growth and in biocontrol (Dowling and O'Gara, 1994). Detailed studies with the siderophore deficient strains proved that production of these metabolites are essential for biological suppression or induced resistance in host plants (De Meyer et al., 1999; Van Loon et al., 1998). Although many soil microbes in culture media produce diverse groups of siderophores, production of these metabolites *in situ* was questioned at times. However, it was demonstrated that siderophores produced by *Pseudomonas* spp. adequately influence microbial interaction (Bakker et al., 1986). This evidence points out an opportunity to antagonize certain soil borne pathogen populations during saprophytic phases of their life cycles through manipulation of the available iron concentration in the rhizosphere.

De Meyer and Hofte (1997) by varying iron nutritional state of the bacterium at inoculation, demonstrated that induced resistance by *Pseudomonas aeruginosa* 7NSK2 was iron-regulated. They established that among the three siderophores produced under iron limitation salicylic acid production and pyochelin were essential for induction of resistance to *Botrytis cinerea* by *P. aeruginosa* 7NSK2 in bean. Transcriptional activity of SA and pyochelin biosynthetic genes was detected during *P. aeruginosa* 7NSK2 colonization of bean. Mutants of same strain lacking SA production have lost their ability to induce systemic resistance. Subsequent studies of De Meyer et al. (1999) demonstrated that in a model with plants grown in perlite, *P. aeruginosa* 7NSK2 induced systemic resistance is equivalent to the inclusion of 1nM SA in the nutrient solution. Leeman et al. (1996) reported that pyoverdin-type siderophore produced by *P. fluorescens* strains WCS 374 and WCS 417 was responsible for ISR in radish against *Fusarium* wilt under iron limitations. Introduction of pchA and pchB gene which encodes for the synthesis of SA in *P. fluorescens* strain P3, render this strain capable of SA production and significantly improved its ability to induce systemic resistance against tobacco necrosis virus (TNV). Similarly *P. fluorescens* strain CHAO naturally produced SA under conditions of iron limitation and induced ISR in tobacco against TNV (Maurhofer et al., 1998).

Studies on the secondary metabolites production revealed that most of the PGPR strains from sugarcane rhizosphere produced one or more metabolites in the culture medium. Purified metabolites exhibited strong antifungal activities against *C. falcatum.* The metabolites *viz.* phloroglucinols and phenazines have been shown to inhibit a wide

range of fungal pathogens in the laboratory. Siderophores exhibit both fungistatic and bacteriostatic effects in the laboratory under controlled conditions of low iron. In the field, these iron-chelating compounds are thought to deprive the pathogen of iron, a limiting essential nutrient (Dowling and O'Gara, 1994). Although the present studies were confined to this level, this information gives scope for further studies on the role of these metabolites production by the bacterial strains and disease suppression in the field. The bacterial strains Pf1, KKM1 and EP1 produce phloroglucinol in the medium. The other effective strains CHAO and VPT4 produced PCA and phloroglucinol. Support for a role of bacterial antibiotics in the biological control of soil borne pathogens has come from a correlation of antibiotic production and pathogen inhibition *in vitro* and disease suppression *in vivo* (Lambert et al., 1987). Role of antibiotic metabolites in disease suppression have been described in studies on chemically or genetically generated mutants defective in the production of specific compounds and mutants for PCA. Rosales et al. (1995) reported production of several antifungal metabolites in the rice associated *Pseudomonas* spp. in the culture and they were found to be highly antifungal to rice pathogens.

Red rot disease is one of the major constraints to sugarcane production in India (Alexander and Viswanathan, 1996; Chona, 1980; Viswanathan and Samiyappan, 1999b). Earlier the authors have demonstrated that the *Pseudomonas* spp. strains were effective against the pathogen under *in vitro* and field conditions (Viswanathan and Samiyappan, 1999a). In our previous studies we have established a significant reduction in red rot disease development in the stalks when the pathogen was challenge inoculated under field conditions. Further, application of these strains in disease-endemic locations reduced disease incidence in red rot susceptible sugarcane cultivars significantly and established that the strains induce systemic resistance in sugarcane against *C. falcatum* (Viswanathan and Samiyappan, 2002). The bacterial strains EP1, KKM1 and VPT4 produce one or more metabolites and such metabolites are highly inhibitory to the pathogen *in vitro.* In addition to their effect of induced systemic resistance (ISR) these strains have shown direct antagonism against the pathogen inoculum surviving in the soil as infected stalk materials. Treating the sugarcane setts with *Pseudomonas* formulations has protected the crop for more than six months under field conditions whereas, in untreated control both pre- and post germination disease development were occurred (Viswanathan and Samiyappan, 1999a). These studies indicate that the metabolites produced by the antagonistic strains are involved either directly or indirectly in the suppression of red rot disease development in sugarcane. Increased plant growth in different sugarcane cultivars after the bacterial strains treatments was achieved under field conditions (Viswanathan et al., 2000). The plant growth promotion achieved might be due to production of metabolites such as IAA and siderophores and elimination of other competitive rhizosphere microbes by the other antibiotic metabolites.

Acknowledgement

The authors are grateful to Linda S. Thomashow, USDA-ARS, Root Disease and Biocontrol Unit, Pullman, USA for providing the reference *Pseudomonas* spp. cultures for the study.

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