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In Vitro Suppression of Soil Borne Pathogenic Fungi and Pyoluteorin Production by *Gluconacetobacter Diazotrophicus*

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ABSTRACT

Gluconacetobacter diazotrophicus an endophytic diazotroph also encountered as rhizosphere bacterium is reported to possess different plant growth promoting characteristics. In this study, we assessed the biocontrol potential of *G. diazotrophicus* under in vitro conditions with different soil borne pathogenic fungus. The possible compounds involved in the biocontrol involves 2,4-diacetylphloroglucinol, pyrrolnitrin and pyoluteorin. Thin layer chromatography analysis revealed that *Gluconacetobacter diazotrophicus* revealed antibiotic, pyoluteorin which helped in the suppression of soil borne pathogenic fungus.

KEY WORDS: *Gluconacetobacter diazotrophicus*, Biocontrol potential, Percent inhibition antibiotics, 2, 4-diacetylphloroglucinol, Pyoluteorin.

INTRODUCTION

An important role in disease suppression has been demonstrated for phenazine-1-carboxylic acid, hydrogen cyanide and 2,4-diacetylphloroglucinol (Keel et al., 1990,1991, 1992; Thomashow and Weller, 1988; Thomashow et al., 1990; Voisard et al., 1989). Pyrrolnitrin and pyoluteorin are further metabolites of *Pseudomonas* strains for which a role in disease suppression has been suggested (Homma and Suzui, 1989; Howell and Stipanovic, 1979, 1980; Jayaswal et al.,1992; Maurhofer et al., 1992).

Gluconacetobacter diazotrophicus originally isolated from sugarcane (Cavalcante and Döbereiner, 1988) was considered to be an endophytic diazotroph. Since then reports on *G. diazotrophicus* from the rhizosphere soils of sugarcane, coffee, ragi and rice reveals it as a prominent rhizosphere dwelling organism (Li and Mac Rae, 1991; Jimenez-Salgado et al., 1997; Loganathan et al., 1999; Muthukumarasamy et al., 2002). Moreover, the detection of *Gluconacetobacter azotocaptans*, a close relative of *G. diazotrophicus* only in the rhizosphere soil of corn strongly suggests its occurrence and survival in soil (Mehnaz et al., in press). The antagonistic potential of *Gluconacetobacter diazotrophicus* against *Colletotrichum falcatum*, the red rot fungal pathogen in sugarcane was first demonstrated by Muthukumarasamy et al. (2000). *F. oxysporum* causes wilt in more than 100 species of crop plants. *F. oxysporum* causes Panama wilt of sugarcane (Buxton, 2008). *F. solani* causes soybean crown and root rot, rot in peas. Ceratocystis fimbriata causes black rot in sweet potato, moldy rot in rubber and wilt in eucalyptus (Roux et al., 2000). Production of plant growth hormones indole-3-acetic acid (IAA) and gibberellins (Bastián et al., 1998) and biocontrol of *Xanthomonas albilineans* – a sugarcane pathogen (Pinon et al., 2002) by *G. diazotrophicus* have also been reported. The purpose of this study wass to determine the anti-fungal potential of *G. diazotrophicus* and to identify its antifungal compound responsible for the inhibition of soil pathogenic fungi.

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MATERIALS AND METHODS

Microorganisms and culture conditions.

G. diazotrophicus PAL5 was kindly provided by Dobereiner (EMBRAPA, Brazil) and the strain L5 from Culture Collection of the Centre of Advanced Studies in Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, India. The *G. diazotrophicus* strains were cultured in LGI medium (g l^{-1} composition: sucrose 100, K_2HPO_4 0.2, KH_2PO_4 0.6, $MgSO_4$. $7H_2O$ 0.2, $CaCl_2$. $2H_2O$ 0.02, Na_2MoO_4. $2H_2O$ 0.002, $FeCl_3.6H_2O$ 0.018, bromothymol blue, 0.5% in 0.2 M KOH 5 ml l^{-1}, pH 5.5) routinely (Cavalcante and Döbereiner,1988). *Fusarium oxsporum, Fusarium solani, Tiarosporella phaseolina,* and *Ceratocystis fimbriata* were obtained from IMTECH, Chandigarh. The fungus was grown in PDA medium (Potato tubers (Peeled) 250 g, Dextrose 20.0 g, Distilled water 1000 ml, pH 5.5 - 6.5, agar 15 g). The reference strain for this study was *Pseudomonas fluorescens* Pf1 obtained from the Department of Plant Pathology, TamilNadu Agricultural University, Coimbatore, India. The *P. fluorescens* Pf1 was grown on King's B agar.

In vitro inhibition of soil borne pathogenic fungus in PDA media.

Antifungal efficacies of *Gluconacetobacter diazotrophicus* strains were tested by dual culture technique (Dennis and Webster, 1971) using PDA medium. A mycelial disc of the fungal pathogen (5mm dia.) was placed at one end of the Petri plate. The bacterial antagonists were streaked 1 cm away from the periphery of the Petri plate just opposite to the mycelial disc of the pathogen. Visual observation on the inhibition of pathogenic fungal growth was recorded after 96 hours of incubation in comparison with the PDA plate simultaneously inoculated with fungal pathogen only as control. (Table 1)

Table1.*Invitro* screening on antagonistic potential of *G. diazotrophicus* cultures on mycelial growth of plant pathogenic fungi in dual culture method on solid media

Fungi	Control	*P. fluorescens* Pf1			*G. diazotrophicus*					
					PAL5			L5		
		Mycelial growth (cm)	Inhibition zone (cm)	Inhibition over control (%)	Mycelial growth (cm)*	Inhibition zone (cm)*	inhibition over control (%)	Mycelial growth (cm)*	Inhibition zone (cm)*	Inhibition over control (%)
Fusarium oxysporum	8.0	4.0	3.0	75.00	4.6	2.0	43.48	4.3	2.3	53.49
Fusarium solani	9.0	3.9	3.2	82.10	4.5	2.1	46.66	4.1	2.5	60.00
Ceratocystis fimbriata	9.0	4.1	2.7	65.85	5.0	1.9	38.00	4.8	2.0	41.00
Colletotrichum falcatum	9.2	4.1	2.6	63.41	5.0	2.0	40.00	4.6	2.6	56.52

Above values presented are the mean of three replications.

In vitro inhibition of soil borne pathogenic fungus in PDA broth.

The PDA broth was prepared and two strains of *Gluconacetobacter diazotrophicus* were inoculated at 1% level. A mycelial disc of the fungal pathogen (5mm dia.) was inoculated at the same time and incubated for 5-7 days after that mycelial weight was measured. (Table 2)

Table 2.*Invitro* screening on antagonistic potential of *G. diazotrophicus* cultures on mycelial growth of plant pathogenic fungi in PDA broth

Fungi	Control	*P. fluorescens* Pf1		*G. diazotrophicus*			
				PAL5		L5	
		Mycelial weight (gm)	Reduction of mycelial weight over control (%)	Mycelial weight (g)	Reduction of mycelial weight over control (%)	Mycelial weight (gm)	Reduction of mycelial weight over control (%)
Fusarium oxysporum	8.833	1.895	3.0	2.890	67.29	2.331	73.62
Fusarium solani	8.632	1.198	3.2	2.312	72.02	2.415	73.20
Ceratocystis fimbriata	8.623	1.341	2.7	1.620	81.22	1.490	82.73
Colletotrichum falcatum	8.453	1.167	2.6	1.418	83.23	1.298	84.66

Above values presented are the mean of three replications.

Percent inhibition (PI).

Percent inhibition of test pathogen by the antagonistic strains was evaluated by dual culture technique (Dennis and Webster, 1971). The radial growth of mycelium in mm was measured and percent inhibition (PI) was calculated.

$$PI = \frac{C - T}{C} \times 100$$

Where, C is the growth of test pathogen (mm) in the absence of the antagonist strain; T is the growth of test pathogen (mm) in the presence of the antagonist strain.

Antibiotics extraction (Cui, 2004).

The *Gluconacetobacter diazotrophicus* strains were grown 96 hours in 1 L of PDA broth. The bacterial suspensions were centrifuged at 5000 rpm for 20 min in order to precipitate the cells. The supernatant was extracted twice with dichloromethane (10:7/supernatant: solvent) and the organic and aqueous phases were mixed gently. The emulsified mixtures were extracted for more than 2h until the complete separation of organic and aqueous phase. The combined dichloromethane organic phases were dried over approximately 5g of anhydrous magnesium sulphate to remove any remaining aqueous residue. The solvent was filtered (Whatman filter paper, 180mm) to discard the magnesium sulphate before being rotary evaporated to dryness. The organic solvent was evaporated by rotary vacuum evaporation at 37 ^{o}C. The residue was resuspended in 1.5 ml of methanol, transferred to a vial and centrifuged at 10000 rpm for 10 min, the supernatant was removed to a clean vial and air dried in a clean cabinet. The residue was redissolved in 1 ml of methanol (at a concentration of 1000 higher than that in the original culture) and sterilized with a 0.22µl filter.

Spotting and developing the TLC plates.

Ten µL of the antibiotic extract from *G. diazotrophicus* PAL5 and L5 were spotted on the plate. The samples were transferred slowly using a micro pipette on the origin as a spot not larger than 5 mm diameter. The spot was allowed to dry each time before applying another. Extra care was taken not to disturb the coating of the adsorbent. The ready made TLC plates were placed in the developing chamber without touching the 'side' of the chamber. The chamber was covered with a watch glass and the TLC was allowed to run undisturbed. When the solvent front touched the 'finishing' line, the plates were removed from the chamber to end the development. For one-way TLC (used for colour reactions and Rf determination), toluene: acetone (4:1; v/v) was used as developing solvent.

RESULTS

***In vitro* inhibition of soil borne pathogenic fungus in PDA media.**

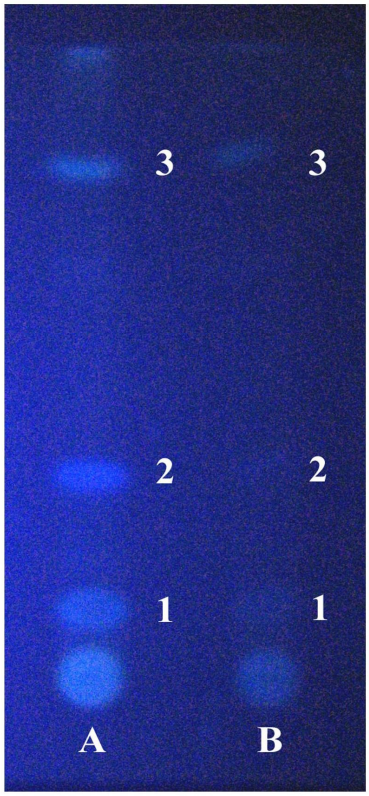
Against *F. oxysporum*, *F. solani*, *C. fimbriata*, and *C. falcatum*, the native isolate L5 of *G. diazotrophicus* reduced the mycelial growth to an extent of 53.49, 60.0, 41.00 and 56.52 % over control, respectively, under *in vitro* conditions. It was followed by PAL5 recording percent inhibition of 43.48, 46.66, 38.00, 50.00 and 40.00 %, respectively. The reference strain *P. fluorescens* Pf1 recorded 75.00, 82.10, 65.85, 71.80 and 63.41 % inhibition over control, respectively (Table 1).

***In vitro* inhibition of soil borne pathogenic fungus in PDA broth.**

Against *F. oxysporum*, *F. solani*, *C. fimbriata*, and *C. falcatum*, the native isolate L5 of *G. diazotrophicus* reduced the mycelial weight to an extent of 2.331g, 2.415g, 1.490g and 1.298 compared to control recording 8.833g, 8.632g, 8.632g and 8.453 of mycelial weight which was 73.62, 73.20, 82.73, 84.66 % less than control, respectively. It was followed by PAL5 (2.890g, 2.312g, 1.620g and 1.418g) with the reduction of 67.29, 72.02, 81.22 and 83.23 % over control, respectively. The reference strain *P. fluorescens* Pf1 recorded 79.68, 86.01, 84.45 and 86.20 % reduction of mycelial weight over control, respectively. (Table 2).

Detection of antimicrobial compound using TLC.

The presence of antibiotics was detected through TLC. The plates were developed in Toluene: Acetone (4:1). Distinct spots corresponding to an R_f value of 0.82, 0.31, 0.13 was visualized under short-wave UV light which could not be visualized under long-wave UV light (Plate 6?? There is no plate in this manuscript). The antibiotics 2, 4-diacetyl phloroglucinol, pyrrolnitrin and pyoluteorin having the R_f values 0.41, 0.53 and 0.31, respectively, were used as standards. The two cultures of *G. diazotrophicus* PAL5 and L5 showed a distinct spot corresponding to an R_f value of 0.31 which was visualized under short-wave UV light corresponds to the antibiotic called pyoluteorin. This confirms the presence of the antibiotic called pyoluteorin in *G. diazotrophicus* PAL5 and L5. (Figure 1).



A - *Gluconacetobacter diazotrophicus* L5
B - *Gluconacetobacter diazotrophicus* PAL5

1 - R_f value (0.13)
2 - R_f value (0.31) - Pyoluteorin
3 - R_f value (0.85)

Figure 1. Detection of antifungal compounds from *G.diazotrophicus* by Thin Layer Chromatography.

DISCUSSION

The phyllosphere microbial community is an open system. Biocontrol specifically in the phyllosphere has been extensively reviewed since 1980 (Andrews, 1990). Blakeman and Fokkema (1982) discussed the potential for biological control of plant diseases in the phylloplane. Cheng *et al*. (1993) reported that a *Pseudomonas* strain P420-4, a *Bacillus* strain B526-7 and a mixture of these were antagonistic to *Alternaria mali* in dual culture studies.

In vitro inhibition studies done both in solid and broth of PDA showed that *G. diazotrophicus* native isolate L5 was effective against the soil pathogenic fungi when compared with the reference strain PAL5 in all the cases. In the case of *Fusarium oxysporum*, *F. solani*, *C. fimbriata*, and *C. Falcatum,* the mycelial growth and mycelial weight was inhibited and reduced effectively by *G. diazotrophicus* native isolate L5 when compared with PAL5. In all the cases, *P. fluorescens* Pf1 showed its full antagonistic capability (Tables 1 and 2).

In present study, the secondary metabolite responsible for antifungal activity was isolated and the metabolites were detected through thin layer chromatography. A fluorescent spot was visualized under UV at R_f value 0.82, 0.32, 0.13. But Cui (2004) reported that antibiotics extracted using Toluene: Acetone (4:1).

In this analysis, *G. diazotrophicus* type strain PAL5 and native isolate L5 produced an R_f value of 0.31. From the standard identity on TLC it was found that the R_f values corresponds to the antibiotic called pyoluteorin and hence it is confirmed that the type strain PAL5 and native isolate L5 produces the antibiotic, Pyoluteorin (Plt). Plt production appeared to be responsible, in part, for the inhibition of *F. oxysporum*, *F. solani*, *C. fimbriata* and *C. falcatum*. In addition two more R_f values were detected in this study, which may correspond to two more new antimicrobial compounds. Purification and identification of the novel compound requires further research.

In conclusion, apart from its biological nitrogen fixation, production of growth regulators, solubilization of heavy metals likes zinc, production of bacteriocins *G. diazotrophicus* strains have the additional property of biocontrol potential against soil borne pathogenic fungus and the biocontrol potential should be explored in-depth to exploit the complete potential of *G. diazotrophicus* as one of the biocontrol agents.

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