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SCREENING AND QUANTIFICATION OF SIDEROPHORE BY GLUCONACETOBACTER DIAZOTROPHICUS STRAINS ISOLATED FROM SUGARCANE

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Abstract

Gluconacetobacter diazotrophicus is an endophytic beneficial bacteria associated with many plants which increase the plant growth and soil fertility by producing different plant growth promoting compounds. In this study, G. diazotrophicus was isolated from various parts of the sugarcane plant materials such as stem, leaf, bud and root. We are assessed from the antagonistic activity of various native isolates of G. diazotrophicus against fungal pathogen such as Fusarium oxysporum, Fusarium solani, Pythium sp, and Colletotrichum falcatum. The native isolate from sugarcane root showed higher inhibitory activity against Fusarium solani by produced salicylate type siderophore. The siderophore production was quantified followed by spectrophotometric method and the maximum siderophore production 177 µg ml-1 was showed by GDSR

Keywords: Gluconacetobacter diazotrophicus, fungal pathogens, Antagonism, Salicylate

1. INTRODUCTION

Rhizosphere is a narrow zone of soil closely associated with plant root system (Walker et al., 2003). The rhizosphere soil was enriched by the exudates from plant root and the rhizosphere microorganisms use this gradient as energy source for them (Dakora and Phillips, 2002). Rhizosphere microorganisms are gives beneficial impact to all the plants except some microbial pathogens that harmful to the plants. Plant growth promotion by rhizobacteria can occur directly and indirectly (Glick, 1995; Persello-Cartieaux et al., 2003). There are numerous ways of plants are using the plant growth promoting bacteria can promote soil quality, e.g. by fixation of atmospheric nitrogen, solubilizing minerals such as phosphorus, production of Siderophores that solubilize and sequester iron, or production of plant growth regulators (Hormones) that enhances plant growth at various stages of development, enormously the fertilization takes place to control the soil growth, it was reported by Yoganathan et al., 2017. Indirect growth promotion occurs when PGPR promote plant growth by improving growth restricting conditions (Glick et al., 1999). This can happen directly by producing antagonistic substances, or indirectly by inducing resistance to the plant pathogens. A bacterium can affect plant growth by one or more of these mechanisms, and also use different abilities for growth promotion at various times during the life cycle of the plant (Glick et al., 1999).

Although a range of different genera and species have been studied, the overwhelming number of researchers has involved the use of Pseudomonas species. But, in addition, they are common rhizosphere organisms and must be adapted to life in the rhizosphere to a large extent (De Wager et al., 1995). There are several aspects reports for the production of metabolites fungi (excluding metal chelators and enzymes) produced by bacteria in vitro that may also have activity by in vivo. These are include ammonia, butyrolactones, 2,4-diacetlyphloroglucinol, HCN, kanosamine, oligomycin A, oomycin A, Phenazine-1-carboxylic acid (PCA), pyoluteorin (Plt), pyrrolnitrin (Pln), viscosinamide, xanthobaccin and zwittermycin A as well as several other uncharacterized moieties (Milner et al., 1996; Keel and Défago, 1997; Whipps, 1997; Nielson et al., 1998; Kim et al., 1999).

Gluconacetobacter diazotrophicus is an endophytic diazotroph also encountered as rhizosphere bacterium is reported to possess different plant growth promoting

characteristics. Fuentes-Ramirez et al. (2001) were able to isolate some new species viz., Gluconacetobacter johannae and Gluconacetobacter azotocaptans from coffee rhizosphere in Mexico. Biocontrol of Xanthomonas albilineans – a sugarcane pathogen by G. diazotrophicus have also been reported (Pinon et al., 2002). Thus the present study was undertaken to find out the antagonistic potentiality of G. diazotrophicus against soil borne fungal pathogens.

2. MATERIALS AND METHODS

Gluconacetobacter diazotrophicus strains were isolated from various parts of sugarcane such as bud, stem, leaf and root then identified by biochemical characteristics also the characteristics were compared with G. diazotrophicus type strain PAL-5 obtained from Tamil Nadu Agricultural University, Coimbatore, Tamilnadu, India. The isolated strains were designated based on the isolation source viz., the strain isolated from bud as GDSB, Leaf as GDSL, stem as GDSS and root as GDSR. The fungal pathogens were obtained from the Department of Plant pathology, Annamalai University, Annamalainagar, Tamilnadu.

Gluconacetobacter diazotrophicus PAL-5 and native isolates were initially grown in two kinds of medium namely LGI (Cavalcante and Dobereiner, 1988) (Having 10% carbon source, normally required for the G. diazotrophicus growth) and in LGIM (Stephan et al., 1992), (having 0.5% carbon source, the lowest concentration for the growth of G. diazotrophicus). Both media were supplemented with 1.32 g/L ammonium sulphate in order to grow non-N_2 fixing cells to build up the population using enough nitrogen. Further, it was multiplied in their respective medium supplemented with 0.132 g/L of ammonium sulphate as N source in order to get the N-fixing cell (Stephan et al., 1992). The cells were allowed to build up to a population $8x10^6$ CFU ml^{-1} and the culture was used as base culture to further study.

Screening of antagonistic bacteria against fungal pathogens

The antifungal activity of G. diazotrophicus strains were assessed by dual culture technique (Dennis and Webster, 1971) using PDA medium. A mycelia disc of the pathogen (5 mm diameter) was placed at one end of the Petri plate. The bacterial strains were streaked 1 cm away from the periphery of the plate just opposite to the mycelia disc of the pathogen. Visual observation on the inhibition of the growth of fungal

pathogens was recorded after 96 hours of incubation in comparison with the PDA plate simultaneously inoculated with fungal pathogens only. 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 the percent inhibition (PI) was calculated.

$$\mathrm{PI} = \frac{C - T}{C} X\ 100$$

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.

Testing of bacterial antagonist for siderophore production

Production of siderophore by bacterial antagonist was assayed by plate assay. The tertiary complex Chrome Azural S (CAS) / $Fe3^{+}$ / hexadecyl trimethyl ammonium bromide (HDTMA) served as an indicator. Forty eight hour old culture of the bacterial isolates were streaked to the succinate medium containing following compounds per 1000 ml, Succinic acid- 4.0g, K_2HPO_4- 3.0g, $(NH_4)_2\ SO_4.7H_2O$- 0.2g, amended with indicator dye. To prepare one liter of blue agar, 60.5 mg of CAS was dissolved in 50 ml of distilled water and mixed with 10ml of iron (III) solution (1mM $FeCl_3.6H_2O$ in 10 mM HCl). While constantly stirring, this solution was slowly added to 72.9mg of HDTMA dissolved in 40ml of water. The resultant dark blue liquid was observed for the formation of bright zone with yellowish fluorescent color in the dark colored medium. It was the indication of production of siderophore. The result was scored either positive or negative to this test (Schwyn and Neilands, 1981) and further, the siderophore production was confirmed by the $FeCl_3$ test. 1 ml of culture filtrate was added in 1-5 ml of ferric chloride solution. The formation of purple color indicated the presence of siderophores (Neilands, 1981).

Detection of nature of siderophore

It was examined by tetrazolium salt test and Carboxylate nature. Instant appearance of a deep red colour by addition of siderophore sample to tetrazolium salt under alkaline

condition indicated the presence of hydroxamate nature (Snow, 1954). Carboxylate nature was detected by Vogel's chemical test where the disappearance of pink color on addition of Phenolphthalein to siderophore sample under alkaline condition indicated the presence of carboxylate nature (Vogel, 1987).

Estimation of siderophore produced by antagonistic bacteria

Siderophore production by the antagonistic bacterial isolates was estimated by the method described by Reeves et al. (1983). PDA broth was prepared for Gluconacetobacter diazotrophicus isolates and dispensed in 100ml quantities in 250ml flask. After sterilization, one ml of standard inoculums of bacterial strains was inoculated into each flask and incubated at 28±2°C for seven days. After seven days of incubation, the broth culture was centrifuged at 10,000 rpm for 20 min and the supernatant was used for the estimation of catecholate type and salicylate type of siderophore.

The pH of the supernatant was adjusted to 2.0 with 1N HCl. To 20ml of supernatant, equal volume of ethyl acetate was added in a separating funnel, mixed well and ethyl acetate fraction was collected. This process was repeated three times to bring the entire quantity of siderophore from the supernatant. The ethyl acetate fractions were pooled, air-dried and dissolved in 5ml of 50 percent ethanol. Five ml of ethyl acetate fraction was reacted with 5ml of Hathway reagent (1ml of 0.1M $FeCl_3$, 1ml of 0.1N HCl in 100ml distilled water + 1ml of 0.1M Potassium ferricyanide). The absorbance was read at 560nm (ELICO SL159 UV-VIS spectrophotometer). A standard curve was prepared using sodium salicylate for the estimation of salicylate type siderophore. The quantity of siderophore synthesized was expressed as $\mu g\ ml^{-1}$of culture filtrate. To measure catechol type of siderophore, five ml of ethyl acetate fraction was reacted with Hathway reagent and absorbance was determined at 700nm (ELICO SL159 UV-VIS spectrophotometer) with 2, 3- dihydroxybenzoic acid as standard. The quantity of siderophore synthesized was expressed as $\mu g\ ml^{-1}$of culture filtrate.

Testing of antagonistic bacteria for the production of hydrogen cyanide

HCN production was determined by using the modified protocol of Miller and Higgins (1970). Bacteria were grown on Tryptic Soy Agar (TSA). Filter paper discs soaked in picric acid solution (2.5g of picric acid, 12.5g of sodium carbonate and 1000ml of

distilled water) were placed in the upper lid of each Petri plate. Dishes were sealed with parafilm and incubated at 28˚C for 48h. A change from yellow to light brown, brown or reddish brown of the discs were recorded as an indication of weak, moderate or strong production of HCN for each strains respectively.

3. RESULTS

Results from in vitro dual culture method for assessment of antagonistic nature of isolates and type strain PAL-5 were showed in figure -1. All the isolated bacterial strains and type strain PAL-5 showed their potential antagonistic activity against all the tested fungal pathogens. Among all the antagonistic strains, GDSR effectively reduce the radial mycelia growth of Fusarium solani (84%) followed by Fusarium Oxysporum (76.49%). The mycelia growth of Fusarium solani was highly reduced by all tested antagonistic strains including type strain PAL-5. Results showed that GDSR was a potential inhibitor of Fusarium solani. Against Pythium sp, all the strains showed least mycelial reduction when compared with other fungal strains but the type strain PAL-5 exhibited maximum mycelia reduction (67.5 %) against *Pythium* sp. The maximum mycelial reduction of 64% against *Colletotrichum falcatum* was observed in plate streaked along with antagonistic strain GDSR.

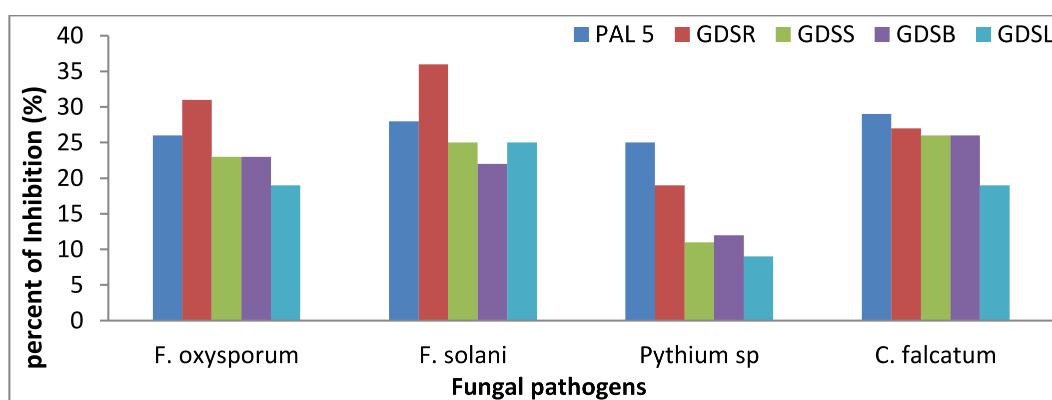

Fig.1 Antagonistic activity of G. diazotrophicus strains

Production of siderophore by tested antagonistic strains determined by Chrome Azural S (CAS) test. All the tested antagonistic strains and type strain PAL-5 gives positive results by produced bright zone with yellowish fluorescent colour in the dark coloured

medium. Further the siderophore production was confirmed by $FeCl_3$ test and G. diazotrophicus all the antagonistic strains produce purple colour that also indicated the presence of siderophores. All the G. diazotrophicus native isolates recorded for the instant appearance of deep red color by the addition of tetrazolium salt to siderophore sample indicating that the nature of siderophore is hydroxamate type, whereas G. diazotrophicus type strain PAL-5 recorded slow appearance of red color. Carboxylate nature was determined by the disappearance of pink color on the addition of phenolphthalein to siderophore sample. All the strains retained indicate pink color only. This indicated the non-carboxylate nature of siderophore produced by G. diazotrophicus strains (table- 1).

Table 1: Siderophore production by G. diazotrophicus cultures

Siderophore assay	Bacterial strains				
	PAL5	GDSR	GDSS	GDSB	GDSL
$Fecl_3$ test	+	+	+	+	+
CAS plate assay	+	+	+	+	+
Hydroxamate test	+++	++	+++	+++	++
Carboxylate test	-	-	-	-	-

+: Positive result, ++: Appearance of light red color

+++: Appearance of deep red color, - : Appearance of pink color

Table 2: Siderophore production (salicylate type) by G. diazotrophicus cultures

Antagonistic strains	**OD value at 560 nm**	**Salicylate type siderophore (µg ml^{-1})**
PAL5	0.26	40
GDSR	0.2	177
GDSS	0.7	40
GDSB	0.89	133
GDSL	0.2	40

Siderophore production by the antagonistic bacterial isolates was estimated and type of the siderophore was tested and the results were showed in table- 2. All the *G. diazotrophicus* strains produced salicylate type siderophore and the maximum salicylate type siderophore production of 177 µg ml^{-1} was recorded by GDSR strain followed by GDSB (133 µg ml^{-1}). Other strains produced 40 µg ml^{-1} of salicylate type siderophore individually in liquid medium. In this experiment, the cultures of *G. diazotrophicus* and type strain were inoculated on TSA plates. This experiment confirmed that the G. diazotrophicus strains and type strains did not produce hydrogen cyanide as there was no change in color of the filter paper disc (table- 3).

Table 3: Production of salicylic acid and HCN by G. diazotrophicus cultures

Antagonistic strains	Salicylic acid	HCN
PAL5	+	-
GDSR	+	-
GDSS	+	-
GDSB	+	-
GDSL	+	-

+: positive, -: negative

4. DISCUSSION

There has been numerous of literature describing the potential uses of plant associated with bacteria which are the agents stimulating plant growth and managing soil by producing numerous enzymes like depolymerize enzymes (Ganesh, et al., 2017) , plant health (Glick, 1995; Hallman et al., 1997; Rovira, 1965; Sturz et al., 2000; Welbaum et al., 2004). The widely recognized mechanisms of biocontrol mediated by PGPR are competition for an ecological niche or a substrate, production of inhibitory allelochemicals and induction of systemic resistance in host of the plant a broad spectrum of pathogens (Bloemberg and lugtenberg, 2001; Glick 1995; Hallman et al.,

1997; Struz et al., 2000) The present study, we have evaluated the mechanism of biocontrol activity of G. diazotrophicus against important fungal plant pathogens and the results were discussed below.

Blakeman and Fokkema (1982) evaluated the possible 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. G. diazotrophicus native isolates were effective against the soil pathogenic fungus when compared with the type strain PAL-5 in all the cases. In the case of Fusarium oxysporum, Fusarium solani, Pythium sp., Colletotrichum falcatum the mycelial growth was inhibited potentialy by G. diazotrophicus native isolates when compared with PAL-5.

The G. diazotrophicus strains have additional properties like usages as bioinoculant to fix N_2 inside the plant system (Jayakumar, 1996; Panneerselvam, 1997; Oliveira et al., 2002), production of growth regulators like production of Indole Acetic Acid (IAA) by 18 strains of A. diazotrophicus analyzed quantitatively by the colorimetric Salkowsky assay (Tang and Bonner, 1947), was as high as 19 to 65 µg ml^{-1} by using the bioassay, HPLC or GC-MS, GA production has been demonstrated in Azotobacter spp (Barea and Brown, 1974; Azcon and Barea, 1975), P. polymyxa, Rhizobium leguminosarum (Atzorn et al., 1988), A. brasilense (Janzen et al., 1999), Acetobacter diazotrophicus and Herbuspirillum seropedicae (Bastian et al., 1999), production of bacteriocins to impede the growth of X. albilineans on solid medium; this factor rapidly diffused in agar medium, a characteristic of lactobacillus-synthesized bacteriocin (Kovenlenko et al., 1999) and solubilization of insoluble zinc compounds (Saravanan, 1999). Fluoresent pseudomonads have revolutionized the field of biological control of soil born plant pathogens. In the past three decades numerous strain of fluorescent pseudomonads have been isolated from the soil and plant roots by several workers and their biocontrol activity against soil born and foliar pathogens were reported. However the research is still lacking to use G. diazotrophicus as a biocontrol agent. Siderophore play a vital role in the suppression of plant pathogen by chelation of Fe thereby creating competition of iron. Siderophore production under iron stress condition confers upon these antagonistic organisms as an added advantage, resulting in exclusion of pathogens due to iron starvation. Although various bacteria siderophore differ in their abilities to sequester iron, in general, they deprive pathogenic fungi of this essential element since

the fungal siderophores have lower affinity (Loper and Henkels, 1997; O' Sullivan and O' Gara, 1992).

In the present study, the G. diazotrophicus strains were plated in CAS medium and the production of siderophore was detected. The type strain of G. diazotrophicus PAL-5 and native isolates produced siderophores of hydroxamate type. Among the cultures, the native isolates of G. diazotrophicus GDSR was found to produce deep red color, whereas the type strain PAL-5 produce light red color in the tetrazolium salt test. The native isolate GDSR produced 177 µg ml^{-1} of silicylate type siderophore. Siderophore are classified on the basis of the chemical functional groups they use to chelate iron. Catechelate-type (phenolate) siderophores bind Fe^{3+} using adjacent hydroxyl group of catechol rings. Fe^{3+} is chelated using nitrogen atom of thiozoline and oxazoline rings in hydroxamate-type siderophores (Crosa and Walsh, 2002). Ferrichrome is the classic hydroxamate-type siderophore. Enterobactin (catechelate type) ferrichrome (hydroxamate type) Bano and Musaurat (2003) reported that Pseudomonas aeruginosa strain NJ-15 produced hydroxamate type of siderophores only. Although diverse group of siderophores are produced by many soil microorganisms in culture media only Schizokinen, a citrate-hydroxamate siderophore produced by Bacillus megaterium and Anabeaena sp., has been purified and identified chemically from the soil (Akers, 1983).

Chincholkar et al. (2000) stated that hydroxamate type of siderophore is produced by both antagonistic fungi and bacteria. Iron being important compound of cells, its deficiency can cause growth inhibition, decrease in nucleic acid synthesis, sporulation inhibition and change in cell morphology. In addition, it also regulates the metabolic processes such as TCA cycle, electron transport chain, oxidative phosphorylation and photosynthesis. The deficit of available iron to pathogens might have resulted in death of the pathogenic organism. Hydrogen cyanide production by certain pseudomonads was found to inhibit the plant root pathogens. In this study, G. diazotrophicus type strain PAL-5 and all the native isolates failed to produce HCN. Arora et al. (2001) reported that none of the rhizobial isolates produce hydrocyanic acid and earlier studies have also reported a very low incidence of cyanogens in rhizobia and in other PGPR. In fact, it has been reported that production of HCN proved to be deleterious to the plant (Antoun et al., 1998). De Britto Alvaraez et al (1995) observed that less than 1% of the 709 isolates obtained from the rhizosphere of tomato were cyanogens. Production of HCN by pseudomonads is associated with biological control of the black root of the

tobacco, but other workers observed that it might have a detrimental effect on plant growth (O' Sullivan and O' Gara, 1992).

CONCLUSION

The present research was under taken with the main objectives of studying the mode of action of G. diazotrophicus against fungal pathogens. Results from this study, the G. diazotrophicus isolates from sugarcane and type strain PAL-5 has potential antagonistic activity against soil born fungal pathogens and its mode of inhibitory nature was determined as production of salicylic type of siderophores. In predominantly, the isolate GDSR showed grater siderophore production and it was naturally protect the plant from fungal pathogens by being from its native rhizosphere-soil environment.

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