Biodegradation of propiconazole by *Pseudomonas putida* isolated from tea rhizosphere

S. Sarkar, S. Seenivasan, R. Premkumar

UPASI Tea Research Foundation, Tea Research Institute, Tamil Nadu, India

ABSTRACT

Biodegradation of triazole fungicide propiconazole was carried out *in vitro* by selected *Pseudomonas* strains isolated from tea rhizosphere. A total number of twelve strains were isolated and further screened based on their tolerance level to propiconazole. Four best strains were selected and further tested for their nutritional requirements. Among the different carbon sources tested glucose exhibited the highest growth promoting capacity and among nitrogen sources ammonium nitrate supported the growth to the maximum. The four selected *Pseudomonas* strains exhibited a range of degradation capabilities. Mineral salts medium (MSM) amended with glucose provided better environment for degradation with the highest degradation potential in strain MPR 4 followed by MPR 12 (72.8% and 67.8%, respectively).

Keywords: tea; propiconazole; *Pseudomonas*; biodegradation; glucose

Tea as a beverage is widely used by human population all over the world. The therapeutic and nutritional value of tea has been well recognized and it is considered as a health drink in today's context. It is indeed desirable to keep this drink completely free from agrochemical residues but this proposition would be unrealistic in view of the serious pest and disease problems on the tea crop. To protect the crop from different pests and pathogens, several fungicides and pesticides are used. Among the various fungicides used, propiconazole is used to protect the crop from blister blight, one of the most lethal diseases resulting in severe crop loss (Premkumar and Baby 1999).

Propiconazole is a broad spectrum triazole fungicide, with systemic and eradicant properties and active against disease caused by Ascomycetes, Basidiomycetes and Deuteromycetes (Nene and Thapliyal 1993). It is active against a number of diseases of cereals (Smith and Speih 1981) and also shows efficacy in a multitude of other crops like peanut, grapes and bananas (Mourichen and Bengnon 1982). Toxicological studies indicated that propiconazole is a product with slight toxicity to mammals. Although environmental exposure to propiconazole is generally presumed to be insignificant due to low application as compared to herbicides and insecticides, the fate of propiconazole became an increasing concern because of it's persistences in soil (Kim et al. 2002), as well as because of its ecotoxicological effects on fish, invertebrates, and algae (Aanes and Bækken 1994, Kaellqvist and Romstad 1994).

Utilization of micro-organisms to metabolically mediate desired chemical reactions or physical processes is a useful general definition of bioremediation (Skladany and Metting 1993). Bioremediation is becoming increasingly adaptable due to its ecofriendliness and is one of the most cost-effective methods compared to physical and chemical remediation methods (Saaty and Booth 1994, Wijesinghe et al. 1992). In the present study, degradation of triazole fungicide propiconazole was studied. For degradation studies, *Pseudomonas* sp. was selected, as it is a well known biodegrader of agrochemicals (Campbell et al. 1995, Johnsen et al. 1996, Kiyohara et al. 1992) and its occurrence was reported earlier in tea rhizosphere (Jayaprakashvel et al. 2006).

MATERIAL AND METHODS

Chemical. For biodegradation studies, reference standard propiconazole with purity of 98.6% [(1-[2-(2, 4-dichlorophenyl)-4-propyl-1, 3-dioxo-

lan-2yl-methyl]-1H-1, 2, 4-triazole)] was obtained from Riedel-de-Haen, Germany. The chemical was dissolved in acetone for further studies. In the present study the degradation of the parent compound (propiconazole) was studied, not its metabolites. The detection and quantification limit ranges for propiconazole in this study were 0.01 ppm and 0.1 ppm, respectively. The average recovery percentage at 0.1 ppm level was 95.5%.

Isolation. The bacterial culture capable of degrading propiconazole was isolated by aerobic shake flask culture in mineral salts medium containing propiconazole (5 mg/kg). The medium contained: 200 mg $MgSO_4$, 900 mg K_2HPO_4, 200 mg KCl, 2 mg $FeSO_4$, 2 mg $MnSO_4$, 2 mg $ZnSO_4$, 1000 mg NH_4NO_3/l. It was adjusted to pH 7.0 by 1N HCl and sterilized by autoclaving at 121°C for 15 min. This medium was inoculated with filtered soil suspension (10% initially and 1%, in subsequent transfer) derived from 10 g tea rhizosphere soil mixed with 150 ml mineral salts medium. Incubation was done at 30–35°C. After incubation, soil suspension was serially diluted and appropriate dilutions were plated on King's B agar medium (King et al. 1954) containing propiconazole. Isolated colonies were subcultured and purified on KB medium containing propiconazole.

Screening. Tolerance levels of the isolates were studied in nutrient agar plates containing different concentrations of propiconazole (5 mg/kg to 10 mg/kg), and the isolates showing the highest degree of tolerance were stored in nutrient agar slants containing propiconazole and stored at 4°C. The selected isolates were screened for their different nutritional requirements.

Nutritional requirements. Effects of different inorganic elements such as carbon and nitrogen sources were studied by replacing them in the basal medium with various sources at 1% level and incubated at 35 ± 2°C. The growth was observed by measuring the absorbance at 560 nm (OD_{560}). The basal medium used was MSM broth.

Biodegradation study. For degradation study, selected isolates were grown on mineral salts medium containing 10 mg/kg propiconazole in 500 ml baffled flask containing 200 ml media. In a separate experiment, media containing glucose and pesticide in MSM media were used for degradation as glucose is easily degradable substance. The cultures were incubated in a rotary incubator shaker (150 rpm) at 30–35°C. After achieving the log phase, the cultures were centrifuged at 9000 rpm for 15 min. After separating the organisms, supernatant was taken in a 125 ml separating funnel and extracted with 50 ml of acetonitrile. After partitioning, the extract was evaporated to near dryness at 65°C using rotary vacuum evaporator and analysed in HPLC (Agilent 1100 series) as per the following conditions: Column: Zorbax Rx C18 (4.6 × 250 mm), Detector: DAD (Diode array detector), Wavelength: 220 nm, Flow rate: 1.5 ml/min, Mobile phase: Acetonitrile (ACN) + Water (80 + 20), Injection volume: 20 µl, Final dilution: 10 ml ACN.

RESULTS AND DISCUSSION

A total of 12 strains (denominated as MPR 1 to MPR 12) were isolated by aerobic culture method. These isolated strains were subcultured and purified in KB medium. The selected isolates were studied for their tolerance level against propiconazole. Results indicated that out of twelve selected isolates, four isolates were capable of tolerating the highest concentration of propiconazole (10 mg/kg), tested in the present study (Table 1). These four isolates (MPR 4, MPR 8, MPR 11 and MPR 12) were studied for their nutritional requirements (Figure 1). Among the different carbon sources, glucose showed the highest growth promoting capacity followed by galactose (Table 2). Among different nitrogen sources, ammonium nitrate exhibited the highest growth-promoting capability (Table 3). The four selected *Pseudomonas*

Table 1. Screening of selected isolates on propiconazole amended medium

Isolates	5 mg/kg	7 mg/kg	10 mg/kg
MPR 1	+++	+	–
MPR 2	+++	–	–
MPR 3	+++	++	–
MPR 4	+++	+++	+++
MPR 5	+++	–	–
MPR 6	+++	++	–
MPR 7	+++	+	–
MPR 8	+++	+++	+++
MPR 9	+++	+	–
MPR 10	+++	–	–
MPR 11	+++	+++	+++
MPR 12	+++	+++	+++

(+++) – good growth; (++) – moderate growth; (+) – poor growth; (–) – no growth

Table 2. Growth on different carbon sources (OD at 560 nm)

Isolates	Glucose	Galactose	Maltose	Starch	Carboxyl methyl cellulose	Xylose	Mannose	Sorbitol	Salicin
MPR 4	1.200	0.875	0.291	0.457	0.456	0.281	0.225	0.413	0.381
MPR 8	1.309	0.782	0.311	0.363	0.347	0.448	0.236	0.548	0.352
MPR 11	1.356	1.025	0.214	0.312	0.531	0.423	0.324	0.521	0.358
MPR 12	1.213	0.914	0.310	0.214	0.412	0.546	0.276	0.312	0.215
SEM ±	0.25	0.23	0.25	0.23	0.24	0.26	0.25	0.24	0.22
CD at 5%	0.57	0.53	0.56	0.52	0.55	0.58	0.56	0.55	0.51

Table3. Growth on different nitrogen sources (OD at 560 nm)

Isolates	Ammonium sulfate	Ammonium nitrate	Ammonium chloride	Sodium nitrate	Urea	Potassium nitrate
MPR 4	0.720	1.613	0.575	0.587	1.035	0.592
MPR 8	0.628	1.885	1.019	0.687	1.071	0.740
MPR 11	1.023	1.426	1.337	0.472	1.102	0.672
MPR 12	1.112	1.692	1.206	0.876	1.062	0.627
SEM ±	0.25	0.27	0.24	0.22	0.23	0.24
CD at 5%	0.57	0.62	0.54	0.50	0.51	0.55

strains used in propiconazole biodegradation study showed a range of degradation capability. Among the two media composition tested, mineral salts medium with glucose provided better degradation environment towards propiconazole than mineral salts with pesticide alone. Out of the four strains tested for their degradation efficiency, strain no MPR 4 showed the highest level of degradation followed by MPR 12. Strain number MPR 4 degraded propiconazole in glucose amended mineral salts medium up to 72.8% in 24 hours whereas in mineral salts medium its efficiency declined to 41.6%. Strain no. MPR 12 could degrade propiconazole up to 67.8% in the presence of glucose but 37.9% in the mineral salts medium (Table 4). Strain number MPR 8 and MPR 11 could degrade propiconazole by 56.2 and 55.9%, respectively in presence of glucose whereas merely 22.7% and 24.2% were reached in MSM medium alone. The higher degradation velocity in glucose-amended medium indicated that glucose played a crucial role in initial growth of the bacteria as compared to control. It may be due to co-metabolism, where addition of easily metabolized organic matter such as glucose increases biodegradation of recalcitrant compounds that are usually not used as carbon

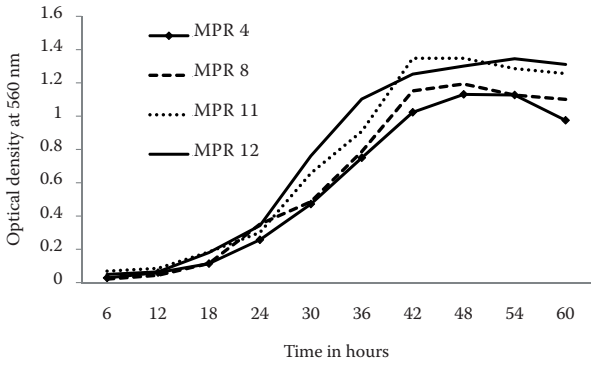

Figure 1. Growth profile of the selected isolates in propiconazole amended medium with glucose

Table 4. Biodegradation of propiconazole (%)

Isolates	Glucose present	Glucose absent
MPR4	72.8	41.6
MPR8	56.2	22.7
MPR11	55.9	24.2
MPR12	67.8	37.9
SEM ±	0.37	0.35
CD at 5%	0.85	0.80

Table 5. Morphological and biochemical characteristics of the selected isolates

Fluorescent pigments on	Isolates			
	MPR 4	MPR 8	MPR 11	MPR 12
Kings B	+ ve	+ ve	+ ve	+ ve
Kings A	– ve	– ve	– ve	– ve
Gram reaction	– ve	– ve	– ve	– ve
Cell morphology	small rods	small rods	small rods	small rods
Oxidase	+ ve	+ ve	– ve	+ ve
Catalase	+ ve	+ ve	+ ve	+ ve
Starch	– ve	– ve	– ve	– ve
Gelatin	– ve	– ve	– ve	– ve
Arginine	+ ve	+ ve	+ ve	+ ve
Citrate	+ ve	+ ve	+ ve	+ ve
Growth at 41°C	– ve	– ve	– ve	– ve

(+ ve) – positive; (– ve) – negative

and energy sources by microorganisms (Prescott et al. 2002). Previous reports suggested the use of glucose as co-substrate increased biodegradation rate (Swaminathan and Subrahmanyam 2002, Movahedin et al. 2006). This process of co-metabolism is finding widespread applications in biodegradation management (Hopkins et al. 1993, Hopkins and McCarty 1995). Degradation of propiconazole by isolated tea rhizosphere microflora showed that these organisms adapted to this chemical because of repeated applications in field. Earlier studies suggested that many soil-applied pesticides are degraded more rapidly following repeated application at the same site (Racke and Coats 1990). Bacterial detoxification of biocides like propiconazole was reported earlier (Herring 1999). A range of environmental factors such as oxygen and temperature are also important for microbial growth rates and pesticide degradation (Bending and Cruz 2007). On the basis of the morphological and biochemical characteristics of isolates (Table 5, Figure 2), the cultures were found to belong to *Pseudomonas putida*, according to Stolp and Gadkari (1981). *Pseudomonas* is a versatile genus and previous reports suggested that this genus could degrade a number of chemi-

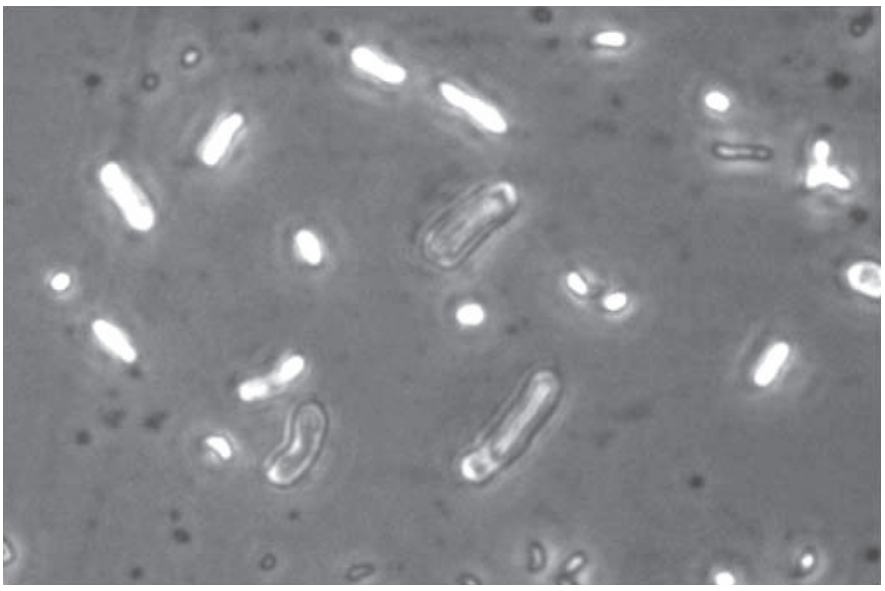

Figure 2. The rod shaped bacteria

cals like pesticides including carbaryl (Vandana et al. 2005), malathion (Hashmi et al. 2004), *p*-nitrophenol and parathion (Douglas et al. 1974), bethoxazin (Wallace and Dickinson 2004) etc.; present study also supported their nutritional diversity. *Pseudomonas* sp. is widely present in soil and can be used to clean up different man-made xenobiotic compounds.

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Corresponding author:

Soumik Sarkar, UPASI Tea Research Foundation, Tea Research Institute, Nirar Dam BPO, Valparai-642 127, Coimbatore Dist., Tamil Nadu, India
phone: + 425 323 53 03, fax: + 425 323 53 02, e-mail: soumiksarkarmib@rediffmail.com