

Evaluation of rhizobacterial isolates for their biocontrol potential of seed borne fungal pathogens of *Jatropha curcas* L.

Meenu Saraf, Urja Pandya, Aarti Thakkar, Palak Patel

Department of Microbiology and Biotechnology, University School of Sciences, Gujarat University, Ahmedabad 380009,
Gujarat, India

Abstract: Seeds attacked by soil borne fungal pathogens are responsible for decrease in germination rate, plant growth and result in losses in yield. The present study aimed to identify fungi associated with *Jatropha curcas* seeds and biocontrol of these pathogens by PGPR strains with their indirect mechanisms. Four fungal species namely *Fusarium oxysporum*, *Macrophomina phaseolina*, *Aspergillus versicolor* and *Aspergillus nidulance* were isolated from *Jatropha* seeds. Ten strains of PGPR isolated from rhizosphere of agricultural crops were studied for their antagonistic activity against seed borne fungal pathogens of *Jatropha curcas* L. After 5 days of incubation, both MSC1 (*Pseudomonas putida*) and MSC4 (*Pseudomonas pseudoalcaligenes*) inhibited the growth of *M. phaseolina* by 90 and 45% respectively. Both the strains showed maximum lytic enzymes production under normal growth conditions. Results revealed that MSC1 and MSC4 treated *Jatropha curcas* offered 29% and 32% disease reductions of *M. phaseolina* respectively over the infected control.

Keywords: Antagonism, Biocontrol, Enzymes, Phytopathogens, *Pseudomonas*

I. INTRODUCTION

Seed borne mycoflora have been found to affect the growth and productivity of crop plants. A seed borne pathogen present externally or internally or associated with the seed as contaminant may cause abortion, seed rot, seed necrosis, reduction or elimination of germination capacity as well as seedling damage. This results in development of diseases at later stages of plant growth by systemic or local infection [1]. The oil plant *Jatropha curcas* L., a multipurpose drought resistance, perennial plant belonging to *Euphorbiaceae* family is gaining a lot of importance for the production of bio-diesel [2]. The seeds of physic nut are a good source of oil which can be used for blending with diesel substitute. Infected *Jatropha* seeds imply an irreversible degenerative change in the quality of seed after it has reached its maximum quality level. The fungi associated with seeds at the harvest stage and under storage bring about several undesirable changes and degradation of seed constituents, thus making the seed unfit for oil extraction, export purpose, consumption or sowing [3,4]. Black rot in *Jatropha curcas* L. plantations by *B. dothidea*, *F. oxysprum*, *M. phaseolina*, *P.longicolla* and *A. alternata* that caused black lesions (soft and rotting) on the stem under the bark and cambium, then they spread to the stems causing shriveling and gummosis of hard wood stems and finally led to the death of the infected plant[5].

Rhizospheric soil is a repertoire of microorganisms that includes beneficial rhizobacteria and pathogenic species that thrive in a nutrient enriched environment. Beneficial rhizobacteria are known to antagonize root pathogens through competition for nutrients and niche, aggressive colonization, parasitism, predation, production of lytic enzymes, antibiotics, bacteriocins, salicylic acid, release of volatiles, surfactants, rhamnolipids and induced systemic resistance [6]. Recent environmentally-friendly cultivation practices emphasize the need to maintain the microbial balance in the rhizosphere by application of plant

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 12, December 2013

growth promoting rhizobacteria (PGPR) and thereby, limit the density of soil borne pathogens to a minimum level for disease control [7]. Also, in disease control programs, the density of biocontrol agents in rhizosphere and efficient root colonization has a clear relationship with suppression of soil borne plant diseases.

Among such beneficial rhizospheric microflora, species of *Pseudomonas* and *Bacillus* are well known for their plant growth promoting potential, aggressive rhizospheric colonization, and antagonistic abilities including the secretion of number of metabolites including antibiotics, volatile hydrogen cyanide (HCN), siderophores, lytic enzymes like chitinases and β 1, 3 glucanases. Pseudomonads have been reported for biological control of different fungal species such as *Rhizoctonia*, *Fusarium*, *Sclerotium*, *Pythium* and *Macrophomina* [8]. Thus, the present work was designed to study the biocontrol efficacy of PGPR (Plant growth promoting rhizobacteria) isolates against fungal pathogens that caused diseases in the seeds of *Jatropha curcas*.

II. MATERIALS AND METHODS

Bacterial isolates

Ten bacterial isolates viz. MSC1, MSC4, B15, B7, B8, K8, K10, K11, K2 and K7 were obtained from the corresponding author's collection section, Department of Microbiology and Biotechnology, Gujarat University, Gujarat, India. They were maintained on Nutrient agar media at 28°C for 2 days. These isolates were already studied for their plant growth promoting potential like phosphate solubilization, siderophore production, IAA production [9].

Isolation of fungal pathogens

Pathogenic fungi were isolated from the infected seeds of *Jatropha curcas* by single spore isolation and maintained on potato dextrose agar (PDA) medium. They were identified based on the colony morphology and lactophenol cotton blue strains techniques which determine the type of reproductive mycelium, conidiophore shape, attachment, color of spore [10].

In vitro antagonistic properties

Antagonistic properties of PGPR isolates were tested against *F. oxysprum*, *M. phaseolina*, *A. versicolor*, and *A. nidulance* on plates having an equal proportion of NAM (Nutrient Agar Medium) and PDA (Potato Dextrose Agar) by dual culture technique [11]. Growth inhibition was calculated by measuring the distance between the edge of bacterial and fungal colonies, and percent inhibition was calculated by following formula:

$$\% \text{ Growth inhibition} = [(C-T)/C] \times 100$$

Where C = Radial growth of fungus in control, T = Radial growth of fungus in dual culture

Cell wall degrading enzyme activities

Chitinase activity was tested on a chitin agar plate containing 1.62 g nutrient broth (Difco, USA), 0.5 g NaCl, 6 g M 9 salts (Difco, USA), 8 g colloidal chitin and 15 g agar per liter; formation of clear halos surrounding growth after 3 day incubation at 30°C indicated positive chitinase activity [12]. Proteolytic activity was determined by using skimmed milk agar (pancreatic digest of casein 5 g, yeast extract 2.5 g, glucose 1 g, 7% skim milk solution 100 ml, agar 15 g dissolved in 1 l distilled water). After 2 days incubation at 28°C, a clear zone around the cells indicated positive proteolytic activity [13]. Cellulase activity was determined on M9 medium agar amended with 10 g of cellulose and 1.2 g of yeast extract per litre of distilled water was used, in which a clear halo after 3 days of incubation of the colonies at 28°C was considered as positive for cellulase production [14].

Enzyme Assays

Chitinase assay : Quantitative Assay for chitinase enzyme production was carried out in 50 ml of chitin medium [15]. Chitinolytic activity of culture was assayed by measuring release of reducing sugar from colloidal chitin by DNSA method. Amount of reducing sugar released from chitin was determined by reading absorbance at 530 nm. One unit of chitinase was defined as the amount of enzyme which produced 1 μ mole glucose equivalent per minute under the assay conditions.

Protease assay: Quantitative protease activity was determined by using skimmed milk containing Nutrient broth [13]. 50 ml medium was incubated with activated bacterial culture and inoculated at 30±2 °C on shaker (150 rpm) for 3 days. Estimation of released tyrosine amino acid was measured by Lowry's Folin Reagent Method. The blue color development was read in spectrophotometer at 700 nm.

Cellulase assay : Cellulase activity was assayed by the determination of reducing sugar released from carboxymethyl cellulose (CMC). For that 0.5 ml of culture supernatant fluid was incubated with 0.5 ml 1% CMC in 0.05 M sodium acetate buffer, pH

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

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4.8 at 40°C for 1 h. The reducing sugar product was assayed by the dinitrosalicylic (DNSA) method [16] using glucose as the sugar standard. Controls for carbohydrate produced from substrate and of the enzyme preparation were included. One unit of cellulase was defined as the amount of enzyme which produced 1 μ mole glucose equivalent per minute under the assay conditions.

Greenhouse experiments: *Jatropha* seeds (*Jatropha curcas* SDAU J1 Chhatrapati), collected from the Regional Research Station S.D. Agriculture University, Sardarkrushinagar, Gujarat, were soaked in 0.02 % sodium hypochlorite for 2 min and washed five times with sterilized distilled water. Seeds were coated with 1 % carboxymethylcellulose as an adhesive. Seed bacterization was done as described by Jha & Saraf [17]. Inoculum of *M. phaseolina* was prepared by method of Singh *et al.* [8]. The earthen pots (24cm x 12cm x 12cm) were filled with sterilized soil. *Jatropha* seeds were sown in six sets of treatment: (i) soil without bacteria and fungus (ii) *M. phaseolina* control (iii) Soil inoculated with MSC1 (iv) soil inoculated with MSC1 and *M. phaseolina* (v) Soil inoculated with MSC4 (vi) soil inoculated with MSC4 and *M. phaseolina*. Growth parameters were recorded after 30 days of inoculation and disease incidence was determined as percentage of the plants showing root rot symptoms. Three replications of each treatment were performed.

III.RESULTS AND DISCUSSION

Isolation and Identification of bacterial and fungal strains: All ten PGPR isolates used were gram negative, short rods, non spore forming, non capsulated and motile rods. Colonies were smooth, translucent, large, low convex. Among all the isolates, MSC1 (*Pseudomonas putida*- accession number GU 553133) and MSC4 (*Pseudomonas pseudoalcaligenes*- accession number GU 564407) were identified in the previous study as good plant growth promoting rhizobacteria [9]. Four fungal phytopathogens were isolated from *Jatropha curcas* seeds on PDA medium by single spore isolation. These four pathogens were identified on the basis of their morphological and microscopical characters (Table 1).

Table 1. Morphological and microscopical characteristics of fungal pathogens from *Jatropha curcas* L. seed.

No.	Morphological characteristics	Microscopical characteristics	Fungal Identification
1.	Colonies are initially white becoming tinged with salmon and lavender at maturity.	Hyphae are septate and hyaline. Conidiophore is short and not branched. Macroconidia produced abundantly, slightly sickle shaped	<i>Fusarium oxysporium</i>
2.	Dark and black appressed mycelia growth.	Microsclerotina are present.	<i>Macrophomina phaseolina</i>
3.	Colonies are light pink to reddish brown.	Hyphae are septate and hyaline. Conidial heads are biserial and loosely radiate. Reduced conidial structures.	<i>Aspergillus versicolor</i>
4.	Colonies are dark green with orange to yellow in areas of cleistothecial production.	Hyphae are septate and hyaline. Conidial heads are columnar and smooth walled. Vesicles are hemispherical, small with metulae and conidia are globose and rough.	<i>Aspergillus nidulans</i>

In vitro antagonistic properties

Fungal growth inhibition

Biocontrol of pathogenic fungi was studied by using ten PGPR on liquid medium. Growth inhibition of all four fungal pathogens ranged from 20 to 90% in liquid medium after five days of incubation (Fig.1). MSC1 showed maximum 60%, 90% and 78% inhibition against *F. oxysporum*, *M.phaseolina* and *A.versicolor* respectively. Whereas, isolate MSC4 and B7 showed 75.6 % and 67.9% respectively against *A. versicolor*. MSC1 isolate also showed maximum inhibition (60%) followed by MSC4 (51.3%) and K7 (50%) respectively against *A. nidulance*. Enhanced plant growth in addition to disease control has been achieved with *Pseudomonas* and *Bacillus* [18, 19]. *Pseudomonas* spp. is reported for the biocontrol of different fungi including species of *Rhizoctonia*, *Fusarium*, *Sclerotium*, *Pythium*, *Erwinia* and *Macrophomina* [20].

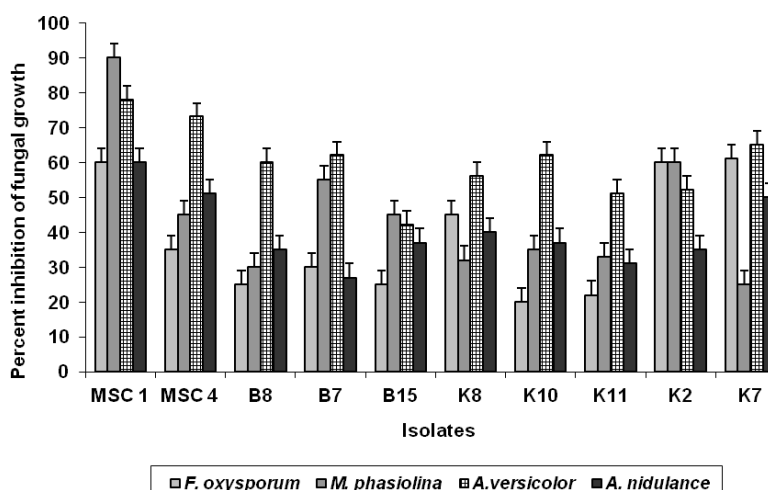


Figure 1. Fungal growth inhibition on liquid medium by PGPR isolates.

Enzyme Assays

Only five isolates formed clear zones around the spot on chitin agar plates, which confirm the chitinase activity. Further, these strains were assayed for their chitinase activity in liquid medium (Fig. 2). Chitinase activity was measured by measuring the amount of releasing sugar after 14 days of incubation. On solid medium zone of diameter was ranged from 5 to 17mm after five days of incubation. Chitinase activity ranged from 0.2 to 0.17U/ml by all the isolates. Maximum activity was observed by isolate MSC4 (0.25U/ml) and MSC1 (0.17U/ml). Both the qualitative and quantitative cellulase production was studied (Fig. 3). Cellulase activity was measured by measuring the amount of releasing sugar after 14 days of incubation. On solid medium zone of diameter was ranged from 5 to 15mm after five days of incubation. Cellulase activity ranged from 0.05 to 0.25 U/ml by all the isolates. Maximum activity was observed by isolates MSC1 (0.25U/ml) and MSC4 (0.15U/ml). Protease activity was measured by measuring the amount of releasing sugar after 14 days of incubation (Fig. 4). On solid medium zone of diameter was ranged from 13 to 25mm after five days of incubation. Protease activity ranged from 95 to 200 U/ml by all the isolates. Maximum activity was observed by isolate MSC1 (200 U/ml).

Chaiharu *et al.* [21] reported screening of PGPR isolates on the basis of lytic enzymes production like chitinase (6%), protease (5%) and cellulase (6%) from total of 220 phosphate solubilizing bacteria that were isolated from different rhizosphere soil in Northern part of Thailand. Pseudomonas strain MIP3 produced maximum chitinase (340 μM/ml) and β 1, 3 glucanase (450 μM/ml) production which caused 87% inhibition of *Aspergillus spp.* reported by Saraf *et al.* [22]. In the present study, growth inhibition of all four fungal pathogens may be attributed to the cumulative effect of all extracellular antifungal compounds including siderophores, HCN, chitinase, cellulase and protease.

Green house experiments: Results of the *in vitro* study showed that among all the ten isolates MSC1 and MSC4 showed maximum inhibition against all the four fungal phytopathogens especially against *M. phaseolina* by producing maximum amount of lytic enzymes. Therefore, these two isolates MSC1 and MSC4 were selected for biocontrol potential against *M. phaseolina* under greenhouse experiments. Maximum stimulatory effect on vegetative plant growth parameters (*viz.* shoot-root length and seedling biomass) was recorded with treatment of MSC1 alone followed by MSC1+ *M. phaseolina* (Table 2 and Fig. 5). Results revealed that *Jatropha* seed treated with MSC1 showed significantly higher root and shoot length (21.14 and

62.94%) whereas two fold and fourfold increase of fresh and dry biomass was observed in *M. phaseolina* infested soil. In *M. phaseolina* infested soil, plant showed rot symptoms when harvested after 30 days. Sclerotia were clearly visible beneath the epidermis of the root and collar region of infected plant and showed 85% of disease incidence. When treated with MSC1 and MSC4, disease incidence was only 29% and 32% respectively. Similar results were obtained with PGPR strains like *Bacillus subtilis* BN1 from the rhizospheres of chir pine (*Pinus roxburghii*; [23], fluorescent *Pseudomonas* GRC2 from potato rhizosphere [24], and *Pseudomonas chlororaphis* SRB 127 from sorghum rhizosphere [25] that showed strong antagonistic effect against *M. phaseolina*, a charcoal rot pathogen of peanut and sorghum.

Table 2. Effect of bacterial seed bacterization on the growth of *Jatropha* under pot experiments

Treatment	Root length(cm)	Shoot length(cm)	Fresh biomass (g)	Dry biomass (g)
MSC1	7.19**	15.15*	7.09**	5.13**
MSC1 + <i>M. phaseolina</i>	6.19*	10.07*	6.86*	4.49**
MSC4	6.88*	12.01**	6.19*	4.04*
MSC4+ <i>M. phaseolina</i>	6.01*	10.02 ^{ns}	5.21*	3.22 ^{ns}
<i>M. phaseolina</i>	5.11 ^{ns}	6.18 ^{ns}	2.89*	1.07*
Control	6.22	7.01	3.85	1.99

Valued are mean of three replicates. * Significant at 5% (P< 0.05 level of ANOVA), ** Significant at 1% (P< 0.01 level of ANOVA), ^{ns} Not significant at 0.05 level of LSD as compared with control.

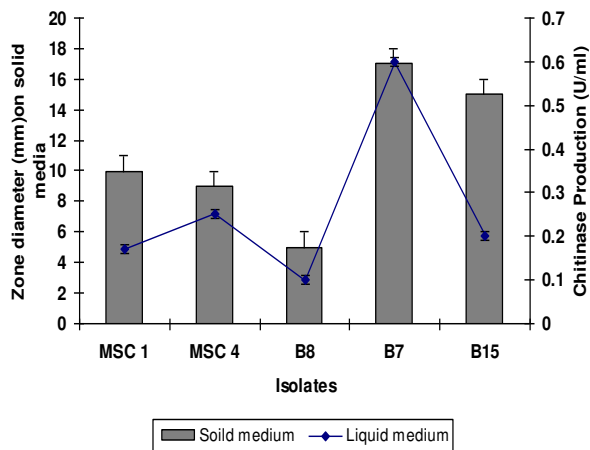


Figure2. Chitinase Production

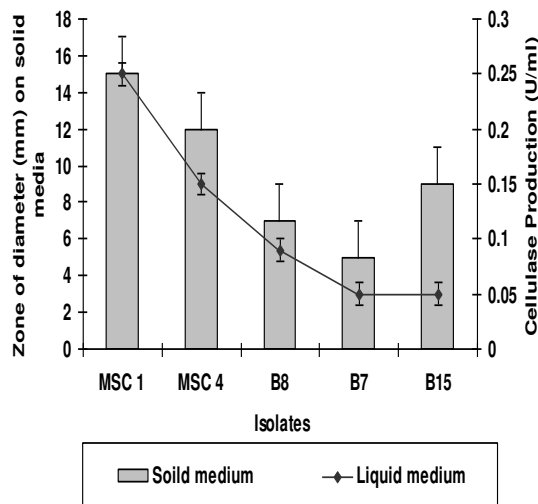


Figure 3. Cellulase Production

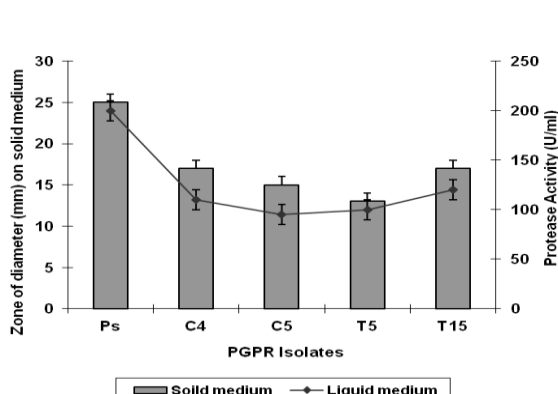


Figure 4. Protease Production

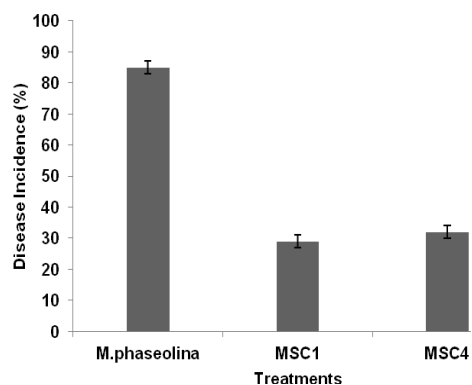


Figure 5. Percent disease inhibitions in various treatments

IV.CONCLUSION

Our findings establish the possible application of antagonistic pseudomonads strains (MSC1 and MSC4) in bioprotection against the seed borne disease as well as growth promotion of *Jatropha curcas*. This study highlights the importance of lytic enzymes (chitinase, cellulase and protease) produced by MSC1 and MSC4 for biocontrol of pathogens. Further investigations have to be carried out to find out the important antifungal compounds that can influence the disease reduction by both the strains.

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BIOGAPHY



Dr. Meenu Saraf, is a professor in the Department of Microbiology and Biotechnology, University school of sciences, Gujarat University, Ahmedabad, Gujarat. Prof Saraf specializes in Microbiology and Biotechnology related to environment and soil.

She started her career as Assistant Professor in Department of Microbiology, Gujarat University, Ahmedabad, Gujarat, India. In 2005 she has been appointed Professor in the same department. She has published more than 40 research papers in journals of National and International reputed and is a leading author of Springer, Heidelberg, Germany by contributing chapters in many books. She has also contributed to Plant Growth and Health Promoting bacteria under the series of Microbiology monograph. Prof Saraf has written popular articles, science communications and reviews for UG students and PG students in Microbiology, Biotechnology and Environment. Recently, she has edited a book on Bacteria In Agribiology: crop Productivity with Springer, Germany.

. Her current research interests include the restoration of marginal and degraded land forms and the influence of stress biotic and abiotic conditions on Plant microbial interactions. She has also worked on isolation and partial purification of various enzymes and metabolites with respect to plant pathology and Biocontrol. Professor Saraf is a fellow of the Biotechnology Society, Life member of Association of Microbiologists of India, Indian Botanical Society, Asian PGPR Society and a member of many other societies like European Biotech Federation, Symbiosis, CAMS etc. She is the recipient of Young Scientist Award of Indian Science Congress Association and the host of INDO-US visiting Professor Award. She has collaborative research projects in University of Aberdeen (UK), University of Waterloo (Canada) and Kentucky State University (USA). Prof. Saraf has travelled several European, North American and South Asian countries on official assignments. She has completed many research projects with government and private agencies and currently has a Biofuel project with British Petroleum International Ltd. Recently she has filed for a patent in this area.