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Chitinolytic and secondary metabolite producing *Pseudomonas fluorescens* isolated from Solanaceae rhizosphere effective against broad spectrum fungal phytopathogens

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ABSTRACT

18 Bacterial isolates were isolated from the Rhizosphere of Solanaceae family namely brinjal, capsicum, chilli and screened for the production of chitinase enzyme. Total of 10/18 isolates were the most potent chitinolytic bacterial species. Of these, 6 isolates showing zone size > 5mm were chosen for further studies. These isolates also showed varied levels of PGPR traits –siderophore, HCN, phosphate solubilisation and IAA. Dual plate assay against few selected soil borne phytopathogens- Alternaria alternata OTA36; Alternaria brassicola OCA1; Alternaria brassiceae OCA3; Collectotrichum gleosporidose OGC1 revealed broad spectrum anti-fungal activity by isolate R. The isolate R was identified as Pseudomonas fluorescens by biochemical test. Concurrent production of siderophore, IAA, HCN, phosphate solubilisation, NH_3 and catalase coupled with anti-fungal activity suggests the plant growth promotion and broad spectrum biocontrol potential of this isolate. Seed bacterization of chilli seeds with Pseudomonas treatment showed 100% germination index and almost 50% reduction in disease incidence by C. gloeosporiodes OGC1 suggesting both the biocontrol and PGPR aspect of the bacteria.

Keywords: *Pseudomonas fluorescens*, chitinolytic, antagonism, PGPR traits, seed bacterization.

INTRODUCTION

The diversity and beneficial activity of the plant-bacterial association and its understanding is important to sustain agro-ecosystems for sustainable crop production [1]. Several bacteria thrive on abundant nutrients in the rhizosphere and some of these possess antagonistic action, which safeguard plants from pathogens and stimulate growth [2]. Biological control of plant diseases using antagonistic microorganisms offers a highly effective, economical and environmental friendly alternative to the use of synthetic pesticides [3]. The mode of action of the antagonistic organisms against various soil-borne plant pathogenic fungi, include biosynthesis of antibiotics, production of hydrolytic enzymes [4], production siderophore and competition for substrates. Successful bacterial antagonists often show a synergistic combination of mechanisms responsible for a successful antifungal interaction. Fluorescent pseudomonads (*Pseudomonas fluorescens*, *P. aeruginosa*, *P. putida*) and closely related species are important antagonistic bacteria present in soil [5]. Some of the fluorescent pseudomonads have currently received world-wide attention due to the production of a wide range of antifungal compounds viz., fluorescent pigments, siderophores, volatile compounds such as hydrocyanic acid (HCN), antibiotics and lytic enzymes. Lytic enzymes (chitinase, β-1,3-glucanase, protease) are responsible for the lysis and hyperparasitism of antagonists against deleterious fungal pathogens. In these mechanisms, chitin, β-1,3-glucan and protein components of the fungal cell wall are digested by these extracellular enzymes. Such bacterial strains have been implicated in the inhibition of plant pathogenic fungi

and deleterious rhizobacteria with a significant increase in root colonization and plant growth. These attributes make fluorescent pseudomonads as the effective biocontrol agents [6-8].

The Solanaceae represent the third most economically important plant taxon, and the most valuable in terms of vegetable crops with agricultural utility [9], representing for more than 3000 species, including the tuber-bearing potato, a number of fruit-bearing vegetables (tomato, eggplant, peppers), ornamental plants (petunias, Nicotiana), plants with edible leaves and medicinal plants.

Colletotrichum is one of the most important plant pathogens worldwide causing the economically important disease anthracnose in a wide range of hosts including cereals, legumes, vegetables, perennial crops and tree fruits [10]. Among these hosts, chilli (*Capsicum* spp.), an important economic crop worldwide [11], is severely infected by anthracnose which may cause yield losses of up to 50%. However, some of the other fungal diseases that lead to wilting in chili are Phytophthora root rot, Verticillium wilt, Rhizoctonia root rot, and Fusarium wilt.

The present study demonstrates the ability of *P.fluorescens* to produce lytic enzymes and secondary metabolites that serve as biochemical weapons against broad spectrum of solanaceae fungal phytopathogens. The protective effect provided by the selected strain on chilli seeds challenged with *Colletotrichum gloeosporoides* when inoculated on seeds is also demonstrated. Since chilli crops are being massively produced, this study may favourably contribute to the development of alternative and sustainable chilli production practices.

MATERIALS AND METHODS

Isolation of rhizospheric bacteria
Bacteria were isolated from solanaceae rhizosperic soils like brinjal, capsicum and chilli grown in Bangalore and Assam by soil dilution method. The different isolates obtained on nutrient agar were screened for chitinase production on chitin agar plates according to [12]. The isolate was routinely maintained on nutrient agar slants at 4˚C. Colloidal chitin was prepared as described by [13].

Characterization of *Pseudomonas* isolates
Identification of the selected *Pseudomonas* was carried out by Biochemical test (Oxidase, Arginine and Gelatin Liquifaction).

Detection of Hydrolytic Enzymes
Chitinase activity was measured according to Chernin et al. [12], protease activity according to Berg et al. [14], and cellulolytic activity on microcrystalline cellulose-containing plates as described by Teather et al. [15]. Lipase was detected qualitatively by fluorescence caused by the fatty acid released due to the action of lipase on olive oil, based on interaction of Rhodamine B with fatty acid released during the enzyme hydrolysis of olive oil [16].

Detection of secondary metabolites
Siderophore Production Siderophore production was tested by growing *Pseudomonas* sp. in the universal siderophore detection medium CAS agar [17].

Detection of the Phosphate Solubilizing Activity Phosphate solubilizing activity was assessed on yeast extract dextrose-$CaHPO_4$ agar plates by measuring the clear zone surrounding the developed bacterial colony, after 7 days of incubation at 30◦C [18].

HCN Production and catalase Hydrogen cyanide production was assayed by the method suggested by Castric (1977) [19]. For catalase detection, bacterial cultures were grown in a nutrient agar medium for 18-24 h at 36±2 °C. The cultures were mixed with appropriate amount of H_2O_2 on a glass slide to observe the evolution of oxygen.

IAA production
Bacterial cultures were grown for 48 h on their respective media at 36±2 °C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Development of pink colour indicates IAA production [20].

Detection of ammonia
Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48–72 h at 36±2 °C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow color was a positive test for ammonia production [21].

Phytopathogens and chilli seeds
The following four phytopathogens obtained as a kind gift from IIHR, Hessarghatta, Bangalore, were used in the study: OCA 1- *Alternaria brassisicola*, OCA 3- *Alternaria brassicae*,OTA36:- *Alternaria alternata*, OGC 1- *Colletotrichum gleosporioides*, 98-01- *Phytophthora capsici*. Remaining phytopathogens were obtained from MTCC Chandigarh: MTCC 4633- *Rhizoctonia solani*, MTCC 1755- *Fusarium oxysporum*. Chilli seeds (Arka Swetha) was obtained as a kind gift from IIHR, Hesarghata, Bangalore.

Antifungal assay
The antagonistic activity of selected *Pseudomonas* isolate against seven phytopathogens was studied by dual culture test. A loopful of 48-h-old culture was spotted on one side of a PDA plate and 6 mm disc of pre grown phytopathogenic fungi inoculated on the other side of the plate. The antibiosis of the selected strains against the fungal pathogens were recorded in terms of diameter of zone of inhibition after every 24 h at 28˚C up to 5 days. Three replicates of each set were taken. The mycelium of *Colletotrichum gloeosporoides* was taken from the zone of inhibition for periodic microscopic examinations [22].

Seed bacterization
Germination efficiency and antagonism against fungal plant pathogens was checked on chilli seeds *in vitro*. The water agar plates were seeded with the following:-
Set 1- Seed control-plain seeds were coated with carboxy methyl cellulose (CMC)
Set 2- Seed coated with CMC and *Colletotrichum* spores
Set 3- Seed coated with CMC and isolate R
Set 4 -Seed coated with both *Colletotrichum* and isolate R

Chilli seeds were surface sterilized successively with sterile distilled water and 0.1% $HgCl_2$. To remove the residual $HgCl_2$ the seeds were washed with sterile distilled water. The isolate was inoculated into Nutrient Broth medium and incubated for 24h at 37˚C. *Colletotrichum* was inoculated onto PDA plates and incubated at 28˚C for 3-4 days. Upon growth of the culture, for set 2 *Colletotrichum* spore suspension was coated. For set 3 the broth with isolate was coated. Similarly for set 4 the CMC coated seed were coated with *Colletotrichum* and isolate (R). The above three sets of treated seeds were seeded onto 1% water agar plates. Plain CMC coated seeds on water agar were used as control. The four sets were monitored regularly for germination and growth. After one week, the sets were observed for germination and biocontrol against *Colletotrichum* coated seeds by the isolate.

RESULTS AND DISCUSSION

Isolation of chitinase positive bacteria
Detection of chitin-degrading bacteria from natural sources such as rhizosphere soil is useful in the isolation of bacteria that produce antifungal or other novel compounds. A high correlation between chitinolysis and production of bioactive compounds has been reported [23-26].

In this study, 18 bacterial isolates were isolated from the Rhizosphere of Solanaceae family namely brinjal, capsicum, chilli and screened for the production of chitinase enzyme. Total of 10 isolates (55.55%) were the most potent chitinolytic bacterial species. Of these, 6 isolates showing zone size > 5 mm were chosen for further studies. Screening of chitinolytic bacteria isolates was carried out by spread inocula of each colony on plates containing a minimal salt medium with colloidal chitin as a sole carbon and energy source. The chitin degrading organism formed colonies of 1-2 mm in diameter, surrounded by clear zones indicating chitinase activity. Microbial chitinolytic enzymes have been considered important in the biological control of many plant pathogens because of their ability to degrade fungal cell walls [27]. Plate 1 shows chtinolytic isolate R on chitin agar plate.

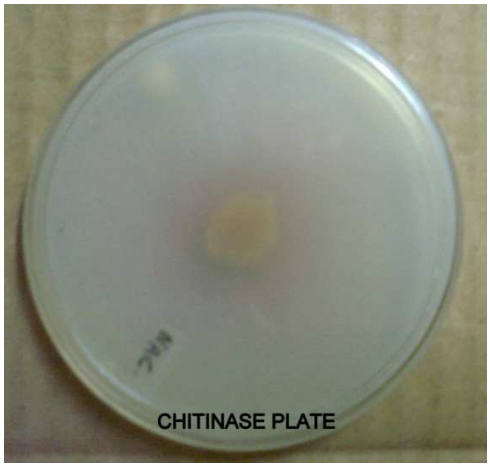
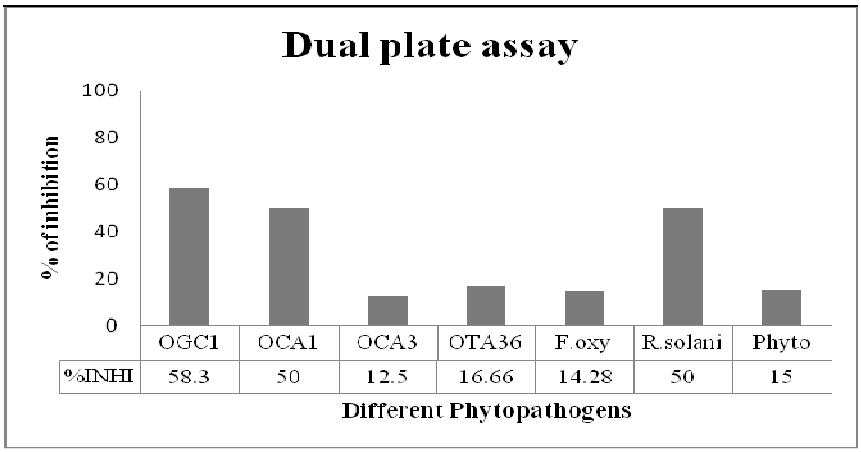


Plate 1 showing chitinolytic isolate R.

Screening for Potential Biocontrol agents against Phytopathogens

Isolate R was found to be an efficient antagonist against all the four phytopathogens tested in dual culture technique (Fig. 1) while none of the other isolates found efficient. Isolate R on co-inoculation with fungal pathogens showed maximum inhibition for phytopathogens in the order: *Collectotrichum gleosporioides* (58.3%), *Alternaria brassicola* (50%), *Alternaria brassiceae* (12.5%), *Alternaria alternate* (16.66%), *Fusarium oxysporum* (14.28%), *Rhizoctonia solani* (50%) and *Phytophthora* (15%).

Fig.1. Antagonism of R v/s phytopathogens determined in terms of % inhibition.

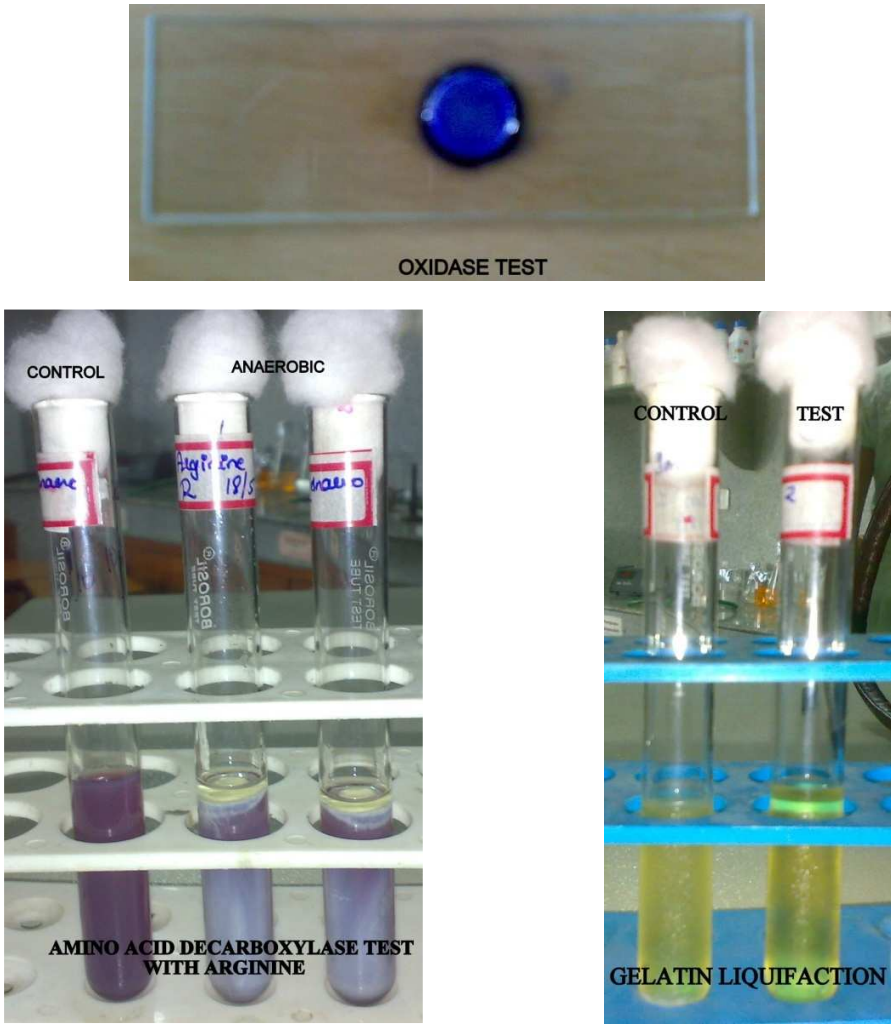


Results are the mean of 05 replicates ± SD. In columns, values with the same letters are not significantly different (P < 0.05 Duncan test)

Identification and characterization of isolate R

Considering the broad spectrum antifungal potential of isolate R, it was used for further studies. The bacterial antagonist R was gram negative, rod shaped and produced yellowish green pigment on King's B medium. It also showed gelatin liquification, was oxidase and arginine dihydrolase positive (Plate 2) and hence was identified as *P.fluorescens* [28].

Plate 2: Biochemical characterization of isolate R

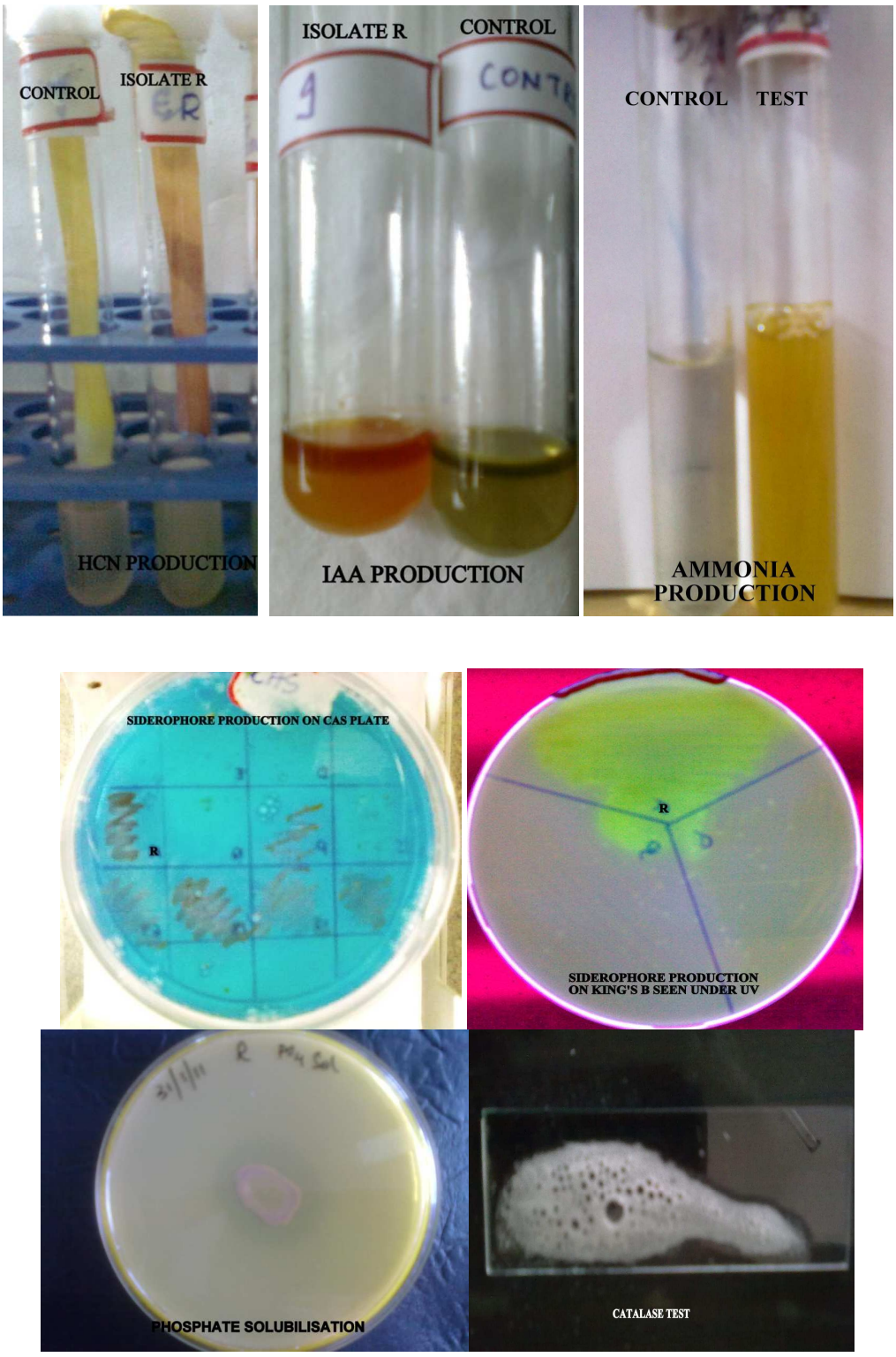


Frommel and Pazos (1993) [29] screened *in vitro* a number of bacterial spp. that inhibited the growth of 11 fungal pathogens, and they found that *Pseudomonas fluorescens*, *P. putida*, *Erwinia herbicola*, *Clavibacter* sp. and *Xanthomonas* sp. reduced the disease incidence caused by *Fusarium* sp. and *Pythium* sp. *Fluorescent pseudomonas* are effective candidates for biological control of soil borne plant pathogens owing to their versatile nature, rhizosphere competence and multiple modes of action besides being endophytic in the plant system including black pepper [23, 30-32].

Determination of PGPR traits & *In vitro* characterizations of the biocontrol mechanism of isolate R

Many species and specific strains of bacteria residing in rhizosphere have been shown to possess plant growth promoting traits and hence they are collectively designated as plant growth promoting rhizobacteria (PGPR) [33]. PGPR enhance plant productivity by a range of direct/ indirect mechanisms. These beneficial effects of PGPR can be either direct or indirect. Direct promotion of growth by PGPR occurs when the rhizobacteria produce metabolites that promote plant growth such as auxins [34], cytokinins [35] and gibberellins [36,37] as well as through the solubilization of phosphate minerals [38]. Indirect growth promotion occurs through the elimination of pathogens by the production of cyanide [39] and siderophores. PGPR beneficial effects have been exploited in many areas including biofertilizers, microbial rhizoremediation and biopesticides [40].

Plate 3: Detection of the various PGPR traits of isolate R.



The isolate R when screened for the PGPR traits showed positive for all the traits – IAA, siderophore, HCN and PO_4 solubilization which may promote plant growth directly or indirectly or synergistically. To investigate the biocontrol

mechanism, the isolate R was tested for production of Non-volatile diffusible antibiotic, production of HCN, and siderophore. R produced non-volatile diffusible antibiotic, HCN and siderophore all of which could be exhibiting varied levels of antagonism on the seven phytopathogens tested (Table 2; Plate 3).

Table 1. Detection of PGPR traits by the isolates

ISOLATES	SIDEROPHORES	IAA	HCN PRODUCTION	PO_4 SOLUBILIZATION
D	-	-	-	-
E	-	-	-	-
I	-	-	+	-
P	-	+	+	+
Q	-	-	-	-
R	+	+	+	+

The finding of multiple PGP activities among PGPR have been reported by some other workers [41]. The R isolate also produced ammonia and catalase. Ammonia production by the PGPB helps influence plant growth indirectly. Bacterial strains showing catalase activity must be highly resistant to environmental, mechanical and chemical stress [42].

Biological control of plant pathogens and deleterious microbes, through the production of antibiotics, lytic enzyme, hydrogen cyanide and siderophore or through competition for nutrient and space can significantly improve plant health and promote growth by increasing of seedling emergence, vigor and yield [43].

Seed bacterization

Treatment of the chilli seeds with pseudomonas R strain showed 100% germination index similar to untreated (Fig 2). The treatment of the seed with co-inoculation of the pathogen with R showed 50% reduction in seed mortality by the treatment as compared to the seed treated with pathogen alone. This treatment also showed 100% germination index suggesting both the biocontrol and PGPR aspect of the bacteria.

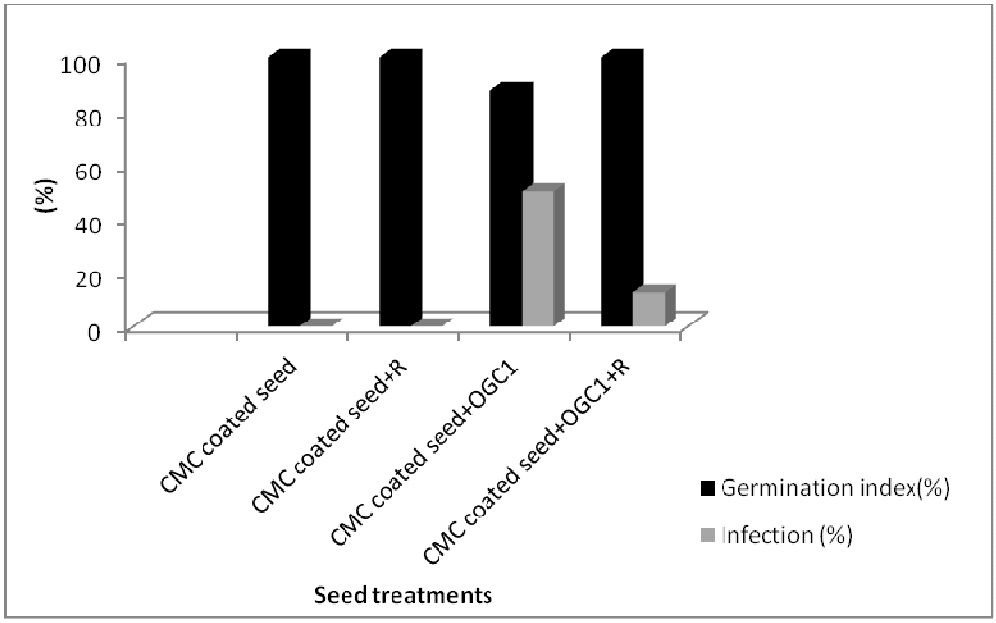


Fig.2. Effect of seed bacterization of chilli seeds with R and *Colletotrichum gloeosporoides* (OGC 1) on germination and biocontrol

Rakh *et al.*, 2001 [44] reported the percent disease control due to *Pseudomonas cf. monteilii 9* treated seeds compared to the untreated check (Positive control), to be in range from 45.45 to 66.67%. These results were

somewhat similar to that got by Kishore *et al.*, 2005 [45] where groundnut seed endophytes *Pseudomonas aeruginosa* GSE 18 and GSE 19 reduced the seedling mortality by 54% and 58%, compared to the control. Our observations also comply with these reports. There are reports of antagonistism of chilli phytopathogen *Pyhtium debaryanum* by soil fungi from chilli rhizosphere [46].

Many investigators have suggested the rhizospheric bacteria *Pseudomonas* spp. as very interesting sources for the identification of antimicrobial compounds and their practical use as biopesticides [47,48, 49].

CONCLUSION

Our study has identified *P.fluorescens* strain possessing multiple mechanism of broad spectrum antagonism and PGP activities which can be explored as one among the best biocontrol agent against Solanaceae phytopathogens.

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