Article AR2293 DOI: HTTPS://DOI.ORG/10.23910/1.2021.2293

International Journal of Bio-resource and Stress Management

August 2021

Crossref

Print ISSN 0976-3988

Online ISSN 0976-4038

IJBSM 2021, 12(4):339-347

Research Article *Stress Management*

Evaluation of *Pseudomonas fluorescens* Strains, Fungicides and Non-conventional Chemicals Against *Botyrotinia ricini* Causing Grey Mold Disease in Castor

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Citation: Basha et al., 2021. Evaluation of *Pseudomonas fluorescens* Strains, Fungicides and Non-conventional Chemicals Against *Botyrotinia ricini* Causing Grey Mold Disease in Castor. International Journal of Bio-resource and Stress Management 2021, 12(4), 339-347. HTTPS://DOI.ORG/10.23910/1.2021.2293.

Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

Conflict of interests: The authors have declared that no conflict of interest exists.

Abstract

A study was made to evaluate the efficacy of *Pseudomonas fluorescens* strains, fungicides and non-conventional chemicals against *Botryotinia ricini*, causing grey mold disease in castor, under *in vitro* conditions. Among the 40 strains isolated from rhizosphere soil samples of different crops across the State of Telangana, India, only eight strains inhibited the growth of *B. ricini* under dual culture technique, of which strains *Pf* 21 (90.56%), *Pf* 23 (88.89%), *Pf* 34 (86.11%) and *Pf* 36 (84.17%) were the most effective. Among the seven chemicals (four fungicides and three non-conventional chemicals) tested for their efficacy, carbendazim followed by propiconazole had significant antagonistic effect against *B. ricini.* Exposure of healthy castor capsules to *B. ricini* and *P. fluorescens* for different time periods revealed that strains *Pf* 34 and *Pf* 36 were effective in completely inhibiting the growth of *B. ricini* and hence these two strains have been identified as effective biocontrol agents, on par with carbendazim, which offer scope for sustainable and integrated disease management of grey mold disease in castor.

Keywords: *Botrytonia ricini*, biological control, castor, fungicides, *Pseudomonas fluorescens*

1. Introduction

Excessive usage of chemicals like fertilizers and pesticides in agriculture has severely affected the environment and the ecosystem. Use of microorganisms as an alternative and replacement of hazardous biochemicals is an important solution for sustainable agriculture. *Pseudomonas fluorescens* is a widely-used plant growth promoter as well as biocontrol agent for the management of plant diseases (Panpatte et al., 2016). It suppresses the disease through various mechanisms such as volatile antibiotics and other auxillary metabolites, siderophores, HCN while also competing with the phytopathogens for niche and nutrients

Article History

RECEIVED on 19th April 2021 RECEIVED in revised form on 04th August 2021 ACCEPTED in final form on 21st August 2021

through induction of systemic resistance in plants against diseases (Bhetwal et al., 2021).

Castor is attacked by several pathogens including fungi, bacteria, viruses and nematodes throughout the world resulting in deterioration of seed quality and market value of the seeds thus causing severe losses to the farmers. Grey mold disease caused by the fungus *Botryotinia ricini* (Godfrey) Whetzel is a major fungal disease in castor. Though the disease is seen wherever the crop is cultivated, it is particularly troublesome for the farmers of South India, especially in Telangana, Andhra Pradesh and Tamil Nadu, where the weather conditions are more favorable for the disease development (Dange et al., 2005). The disease is responsible for disease epidemics and yield losses in Telangana state of India (Arutselvan et al., 2020).

Spike, the economically important part in castor plant, is the primary site of grey rot infection. At later stages, infection is spread to the leaves and stem. Sussel et al. (2009) reported that if the infection occurs during flowering stage, seed filling gets affected resulting in the spike without (or less) capsule formation, thus posing great economic loss to farmers. Continuous cyclonic rainfall and high humidity are very congenial to the development of grey mold in castor (Wilcox and Seem, 1994). Sussel et al. (2011) observed a high correlation between the temperature and duration of leaf wetness with gray mold disease incidence and severity.

Host plant resistance is an effective management strategy and researchers have been attempting to develop resistant cultivars against gray mold. So far, only cultivars with partial resistance and promising accessions (Lima and Soares, 1999; Anjani et al., 2004; Anjani and Raoof, 2010) have been identified. Cultural practices such as selection of good seed, sowing time, removal of infected plant debris and weed hosts, adoption of optimum spacing have been reported to manage the disease (Prasad, 2016; Gahukar, 2018; Soares and Cumagun, 2012, Soratto et al., 2012). Though fungicides such as propiconazole, carbendazim, azoxystrobin and iprodione were proved to be effective in controlling mold (Bezerra, 2007; Sudhakar et al., 2010; Bhat and Rajasri, 2015) the disease can be controlled only with right timing and dosage of application.

Biocontrol agents such as *Trichoderma viride* and *T. harzianum* were effective in inhibiting the mycelia growth of *B. ricini* (Bhattiprolu, 2008). Raoof et al. (2003) reported that *T. viride* (10^6 spores ml^{-1}) application reduced the disease by 45% in detached spikes. Navaneetha et al. (2015) reported disease reduction of 65% on diseased spikes upon treatment with *T. harzianum* and *T. asperillum*. Simionato et al. (2017) and Gao et al. (2018) demonstrated the potential of *Pseudomonas* species in suppressing *B. cinerea* causing gray mold in strawberries, tomato and grapes Indira et al. (2004) reported *T. viride*, *T. harzianum*, *T. hamatum*, *T. koeningii* and *P. fluorescens* as potentially active biocontrol agents against gray mold disease in sorghum. Nevertheless, chemicals are also useful for farmers for immediate disease management. In castor, there is a requirement for newer or alternate disease controlling agents which can act faster and impart efficacy under field conditions. In view of this, an attempt was made to identify biocontrol agent, fungicides and non-conventional chemicals that are effective against *B. ricini* under laboratory conditions.

2. Materials and Methods

2.1. Isolation of the grey mold pathogen B. ricini

The pathogen was isolated on Potato Dextrose Agar (PDA) medium from grey mold infected capsules of castor plants collected from Regional Agricultural Research Station, Palem, Nagarkurnool in Telangana State, India during 2015–16 and maintained at 25°C for three to four days. The culture was further purified by single spore isolation and pure culture of the fungus was maintained on PDA slants and stored at 5°C for further use (Prasad and Bhuvaneshwari, 2014).

2.2. Isolation of P. fluorescens from rhizosphere soil

Soil samples from the rhizosphere of different crops such as castor, sorghum, groundnut, redgram and cotton were collected during 2015–16 from different districts of Telangana State, India. Thirty-day old plants were selected and uprooted and the roots with enclosed soil were placed in plastic bags and stored at 4°C. For the isolation of bacteria from the rhizospheric soil, 10 g of soil was mixed with 90 ml of distilled and sterilized water. Then serial dilution of the suspension (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) was made. Around 0.1 ml from each dilution was grown on Kings B media (peptone 20 g, dipotassium hydrogen phosphate 2.5 g, glycerol 15 ml, magnesium heptahydrate 6 g, agar-agar 15 g, distilled water 1 l) and incubated at 28°C until the bacterial colonies were formed. The plates were then viewed under U.V transilluminator and fluorescent *Pseudomonas* colonies were identified and maintained for further use (Lamichhane and Varvaro, 2013).

2.3. In vitro antagonistic activity of P. fluorescens against B. ricini

P. fluorescens strains were tested against *B. ricini* grown in Petri plates containing PDA medium by dual culture plate technique (Islam et al., 2018). *P. fluorescens* strains were streaked at a distance of 5 cm from the agar discs (5 mm in diameter) of *B. ricini* placed in the middle of the plate. The culture plates were sealed with plastic wrap and later the plates were incubated for eight days at 28°C. A control plate of *B. ricini*, without bacterial isolates was maintained and fungal growth was measured daily during the eight days of study. Zone of inhibition was measured (mm) from the edge of the mycelium to the margin of the each bacterial colony after 5 days of incubation.

2.4. Characterization of P. fluorescens strains for antagonistic traits

Preliminary identification of the isolates was based on colony

formation, colour, pigmentation, shape and Gram staining. The strains were also characterized for production of ammonia, indole acetic acid and siderophores.

2.4.1. Production 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 colour was a positive test for ammonia production (Trivedi et al., 2008).

2.4.2. Production of indole acetic acid

Indole acetic acid (IAA) production was detected as described by Brick et al. (1991). 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 concentrated 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 indicated IAA production.

2.4.3. Siderophore production

Siderophore production was carried out in Kings B medium using blue agar plates containing chromazurol S dye (Schwyn and Neilands, 1987). Orange color halos around the colonies on blue were indicative for siderophore production.

2.5. In vitro efficacy of fungicides and chemicals against B. ricini

Four fungicides (carbendazim, mancozeb, carbendazim+ mancozeb and propioconazole) and three chemicals (oxalic acid, salicylic acid and zinc sulphate) were evaluated for their efficacy on mycelial growth of *B. ricini* by poisoned food technique (Schmitz, 1930). Each fungicide and chemical was dispensed separately in molten sterilized PDA medium at seven concentrations (300, 500, 700, 900, 1000, 2000 and 3000 ppm), obtained by adding sterile water to the stock solutions. Five mm mycelia disc, taken from one week old culture of the *B. ricini*, were asceptically placed in the center of solidified poisoned PDA. Five replications were maintained for each concentration. The Petriplates were incubated at 24°C and observations of the mycelial growth of *B. ricini* were recorded after seven days of incubation. The growth of the fungus on non-poisoned PDA served as a control. The % inhibition in the growth due to various fungal treatments at various concentrations was computed as follows:

Mycelial growth inhibition (%)=[(dc-dt/dc)] x 100 (%).

where, dc=Average diameter of fungal colony in control, and

dt=Average diameter of fungal colony in treatment group

2.6. Efficacy of P. fluorescens against B. ricini using detached capsule technique

Based on *in vitro* experimental data, five strains of *P. fluorescens* that were effective in inhibiting the mycelial growth of *B. ricini* along with the most effective fungicide (or chemical), were tested for their efficacy against *B.ricini* using detached capsule technique (Prasad et al., 2016). Fruit capsules of 15–20 days old were detached from healthy castor spikes, surface sterilized and dipped in a suspension of *B. ricini* and bioagent/fungicide for 0, 1, 4, 6 and 12 hours. For each treatment, four capsules were maintained at 20–25°C and 90% relative humidity in petri plate for a week in three replications. Capsules treated with *B. ricini* were positive control and uninoculated capsules constituted negative control. The number of capsules infected was recorded each day after the appearance of first symptom.

3. Results and Discussion

3.1. Isolation of the grey mold pathogen B. ricini

B. ricini was isolated from grey mold infected castor plants collected from Regional Agricultural Research Station, Palem, Nagarkurnool in Telangana State (Figure 1). The fungus produced pluffy mycelial growth on PDA without sporulation. This is in partial agreement with the observations made by Prasad et al. (2016) who also reported pluffy mycelia growth but with enhanced sporulation of *B. ricini* on oat meal agar medium.

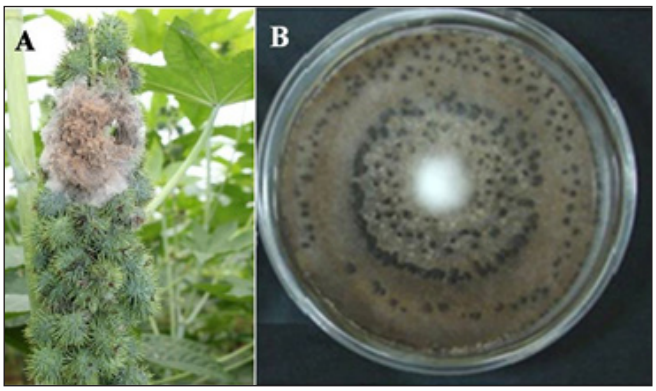

Figure 1a: Castor spike showing grey mold infection. b: Pure culture of *Botryotinia ricini*

3.2. Isolation of P. fluorescens from rhizosphere soil

Forty strains of *P. fluorescens* were isolated from the rhizosphere soil samples of castor, cotton, groundnut, rice and redgram crops in Nagarkurnool district, Telangana State. This is similar to the results reported in rice where thirty *P. fluorescens* isolates were collected from rhizosphere soils (Reddy et al., 2007). The present reporting of 40 *P. fluorescens* assumes significance because there are limited reports on evaluation of so many different isolates of *P. fluorescens against B. ricini.* Though there are several studies on use of other biological control agents like *Trichoderma* spp. to control gray mold in castor (Bhattiprolu and Bhattiprolu, 2006; Chagas, 2009; Raoof et al., 2003; Tirupathi et al., 2006), there are limited studies on use of *P. fluorescens.* Raoof et al. (2003) reported the use of both *P. fluoresens* and *T. viride* to manage *B. ricini,* however, single isolates were evaluated in the study compared to the forty isolates used in this study.

Navaneetha et al. (2015) reported the use of *B. ricini* using *T. harzianum* and *T. asperellum* isolates. Bhattiprolu and Bhattiprolu (2006) evaluated the effectiveness of a single isolate of *T. viride* against *B. ricini* as influenced by culture medium pH, incubation temperature, fungicides and exposure to mutagenic agents (gamma rays).

3.3. In vitro antagonistic activity of P. fluorescens against B. ricini

Among the 40 strains of *P. fluorescens* tested for their antagonistic activity against *B. ricini*, only eight strains (*Pf* 3, *Pf* 4, *Pf* 21, *Pf* 22, *Pf* 23, *Pf* 24, *Pf* 34 and *Pf* 36) showed zone of inhibition against *B. ricini*. Maximum inhibition (90.56%) was recorded by *Pf* 21 followed by *Pf* 23 (88.89%). These two strains were isolated from rhizosphere soils of rice and groundnut respectively. Strains *Pf* 34 and *Pf* 36 recorded 86.11% and 84.17% inhibition respectively. Minimum inhibition was observed with *Pf* 3, *Pf* 4, *Pf* 22 and *Pf* 24 (Figure 2). Interestingly *Pf3* and *Pf4* were isolated from the rhizosphere soils of castor crop (Table 1). *P. fluorescens* strains were proved to be effective in inhibiting the growth of *B. ricini*. The results of this study were in conjunction with the earlier reports on the effect of *P.fluorescens* against *B.ricini* in castor (Singh, 2014). In castor, in addition to *P. fluorescens,* the importance of *Trichoderma* spp. in integrated disease management against grey mold weas reported by Navaneetha et al. (2015). They demonstrated the effectiveness of *T. harzianum* and *T. asperellum* isolates against *B. ricini* by 55–65%. Similarly, Bhattiprolu and Bhattiprolu (2006) showed 60% inhibition of *B. ricini* using poisoned food technique. A gamma ray mutant isolate of *T. viride* TV4 obtained the highest inhibition of *B. ricini* of about 88% when compared to the wild isolate (Bhattiprolu, 2008). In rice, Reddy et al. (2007) reported one isolate (Pf 003) of *P. fluorescens* among the 30 isolates which effectively inhibited the growth of four rice pathogens viz., *Magnaporthe grisea, Drechslera oryzae, Rhizoctonia solani* and *Sarocladium oryzae* by 62 to 85%.

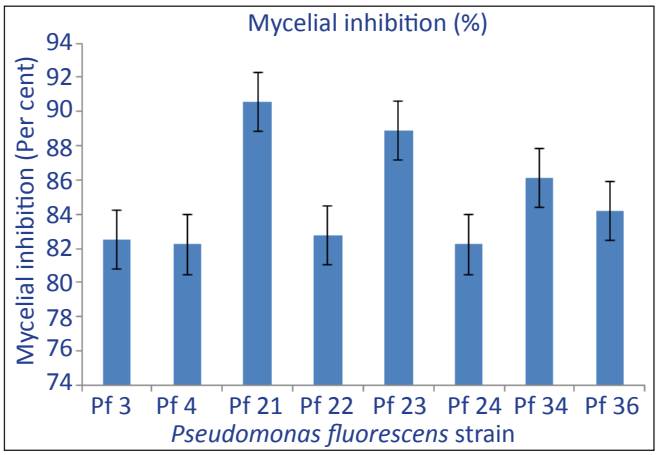

Figure 2: Antagonistic potential of *Pseudomonas fluorescens* against *Botryotinia ricini* under *in vitro* conditions

3.4. Characterization of P. fluorescens strains for plant growth promoting traits

All the eight strains *i.e., Pf* 3, *Pf* 4, *Pf* 21, *Pf* 22, *Pf* 23, *Pf* 24, *Pf* 34 and *Pf* 36) took about 24 hours to establish their growth on kings B agar medium. Colonies were dull white in color, small to medium in size, smooth and glistening with yellowish green to light green pigmentation. The strains were Gram negative, small, single isolated rods without sporulation. The strains were tested for antagonisitic properties *viz.,* HCN, ammonia, IAA and siderophore production. Strains *Pf* 21 and *Pf* 34 tested positive for HCN production (Table 1; Figure 3a).

Similar results were reported by Singh et al. (2015), where in change in color after incubation from yellow to light brown indicated weak HCN production and change in color from yellow to reddish brown indicated strong HCN production. They reported that isolates PSF 1, PSF 3, PSF 8 and PSF 12 produced highest HCN. Bano and Mussarat (2003) reported efficient HCN producing *Pseudomonas aeruginosa* strain NJ-15 as a potential biocontrol agent. Bhoonobtong et al. (2012) also reported the anti-microbial activity of *P. aeruginosa* isolated from medicinal plants in Thailand. All the eight isolates tested positive for ammonia production (Table 1). Four isolates *viz.,*

Table 1: Biochemical characterization of *Pseudomonas fluorescens* strains isolated from different crops cultivated in Nagarkurnool district of Telangana State

Strain	Crop	Location (Mandal)	Biochemical characterization			
			HCN	Ammonia	IAA	Siderophore
Pf 3	Castor	Palem (Bijnepally)	-	+	+	-
Pf 4	Cotton	Palem (Bijnepally)	-	+	+	-
Pf 21	Rice	Edirepally (Thimmajipet)	+	+	-	+
Pf 22	Castor	Nagarkurnool	-	+	+	-
Pf 23	Groundnut	Vattem (Bijnepally)	-	+	-	+
Pf 24	Redgram	Palem (Bijnepally)	-	+	+	-
Pf 34	Groundnut	Sudhakal (Kalwakurthy)	+	+	-	+
Pf 36	Groundnut	Tadoor (Tadoor)	-	+	-	+

Pf 3, *Pf* 4, *Pf* 22 and *Pf* 24 tested positive for IAA (Figure 3b) and four isolates *viz.*, *Pf* 21, *Pf* 23, *Pf* 34 and *Pf* 36 tested positive for siderophore production (Figure 4). Similarly, fluorescent *Pseudomonas* strains were isolated from the rhizosphere soils of various crops namely castor, redgram, rice, greengram, sunflower, potato etc. and characterized for their morphological, cultural and biochemical attributes (Gupta et al., 2002; Bhatia et al., 2008).

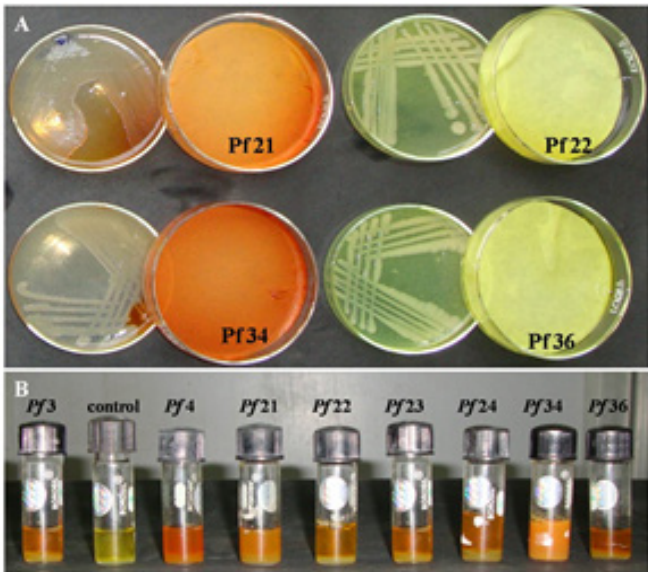

Figure 3: Hydrogen Cyanide production (a) and ammonia production (b) by *Pseudomonas fluorescens* strains isolated from rhizosphere soils of Telangana State

3.5. In vitro efficacy of fungicides and chemicals against B. ricini

Four fungicides tested effectively inhibited mycelial growth of *B.ricini*. There was a significant decrease in mycelial growth with increase in the concentration of fungicide (Table 2). Carbendazim completely inhibited (100%) the growth of *B. ricini* at all the tested concentrations including low concentrations of 300, 500, 700, 900 and 1000 ppm compared to the recommended dose of 1 g l^{-1} (Yasmeen et al., 2003) Interestingly, the combination fungicide *viz.*, carbendazim+mancozeb recorded complete inhibition at concentration 700 ppm and above which is much lower than the recommended dosage of 1.5 g l^{-1}. Propioconazole was also toxic to *B. ricini* with a significant inhibitory effect at all concentrations and showed complete inhibition of *B. ricini* at concentrations 2000 and 3000 ppm. All the three tested chemicals showed significant inhibition of *B. ricini*, with oxalic acid showing complete inhibition at 700 ppm and above. Salicylic acid and $ZnSo_4$ recorded no growth at 2000 and 3000 ppm concentration with negligible growth observed up to 1000 ppm concentration (Table 2). Niki (2003) observed that carbendazim was effective in inhibiting

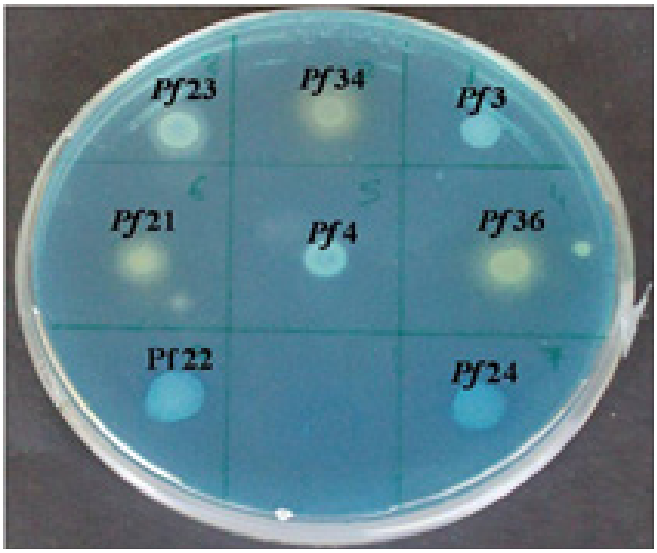

Figure 4: Siderophore production by *Pseudomonas fluorescens* strains collected from rhizosphere soils of Telangana State

Table 2: Efficacy of different fungicides and chemicals against *Botryotinia ricini* under *in vitro* conditions

Fungicides/Chemical	% inhibition of mycelial growth						
	Concentration (ppm)						
	300	500	700	900	1000	2000	3000
Carbendazim	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Mancozeb	82.20	100.00	100.00	100.00	100.00	100.00	100.00
Carbendazim+Mancozeb	84.40	85.40	100.00	100.00	100.00	100.00	100.00
Propiconazole	80.00	82.30	96.77	98.70	99.10	100.00	100.00
Oxalic acid	77.30	78.70	100.00	100.00	100.00	100.00	100.00
Salicylic acid	62.20	64.20	70.00	72.00	81.10	100.00	100.00
$ZnSO_4$ $7H_2O$	59.30	60.00	68.20	77.80	85.50	100.00	100.00
Sterile water (control)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SEm±	0.83	1.17	0.50	0.69	0.68	0.00	0.00
CD (*p*=0.05)	2.46	3.46	1.49	2.04	2.02	0.00	0.00

Botrytis sp. under glass house conditions. Simiarly, Bezerra (2007) also reported that carbendazim was effective against grey rot under controlled conditions. Chagas (2009) reported that carbendazim, tebuconazole, iprodione and procymidone were highly effective against grey rot under *in vitro* and *in vivo* conditions. Fungicides such as armure, thiram, edifenphos and carbendizam and their mixtures were proved effective against *B. ricini* mycelia growth rate and spore germination under *in vitro* conditions. Haenssler et al. (2000) observed that fenhexamid was effective against grey rot under green house conditions. In addition to fenhaxamid, newer fungicides namely pyrimethanil, fludioxonil, fluazinam etc. are also being used along with carbendizam (Couderchet, 2003).

3.6. Efficacy of P. fluorescens and fungicides against B. ricini using detached capsule technique

Symptoms of *B. ricini* infection characterized by grayish cottony mycelial growth appeared on castor capsules on 3 days after inoculation (DAI). By 6 DAI, the infected capsules were fully covered by the fungus. In 0 hour treated set, there was no infection of *B. ricini.* Similarly, in uninoculated control (without *B. ricini*) and only *P. fluorescens* (without *B. ricini*) treated samples, the capsules appeared healthy without any pathogen infection. Overall, increased exposure time to *P. fluorescens* and carbendazim reduced *B. ricini* infection. Capsules treated with carbendazim for 1 hour recorded 25% infection by 6 DAI compared to zero infection in capsules exposed for 4, 6 and 12 hours (Figure 5). Within a given exposure time, *P. fluorescens* strains differed in their efficacy to inhibit *B. ricini* infection on castor capsules. In castor capsules exposed for 1 hour to *P. fluorescens* and *B. ricini*, strain *Pf* 36 recorded 50% infection by 6 DAI compared to 100% infection for *Pf* 22. In capsules exposed to *P. fluroescens* and *B. ricini* for 6 hours, *Pf* 21, *Pf* 34 and *Pf* 36 recorded 50% capsule infection compared to 75% infection for *Pf* 23 and *Pf* 22. Both *Pf* 34 and *Pf* 36 were effective in completely controlling *B. ricini* growth on castor capsules when exposed for 12 hours followed by strain *Pf* 21 with 25% infection. Strain *Pf* 22 was least effective with 75% capsules infected with *B. ricini* (Figure 5). Raoof et al. (2003) reported that *Trichoderma* spp. and *P. fluorescens* controlled castor gray mold to an extent of 40–50% under artificial inoculation conditions.

The evaluation of antifungal activity of different fungicides *in vitro* condition is important for integrated disease management where limited and appropriate quantity of agrochemical should be used for achieving eco friendly management of grey rot. The effectiveness of *P. fluorescens* strains in inhibiting the growth of *B. ricini* has been clearly demonstrated with *Pf* 34 and *Pf* 36 being the most effective strains. Both the isolates tested positive for siderophore production. *P. fluorescens* secretes a hydroxamate-type siderophore which was effective in controlling *Macrophomina phaseolina* in groundnut (Gupta et al., 2002) through their ability to chelate and reduce the amount of ferric ions available in rhizosphere, competing with the plant pathogens and thus reduce their ability to colonize

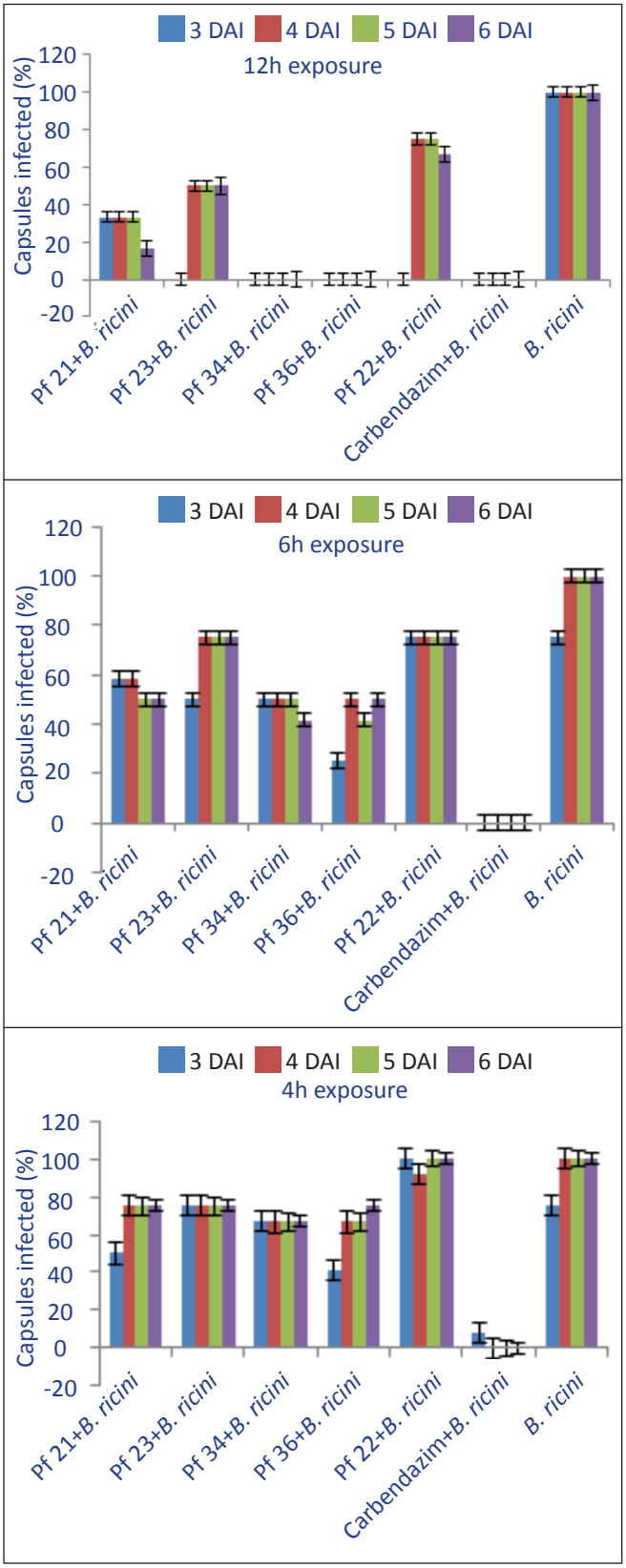

Figure 5: Effect of *Pseudomonas fluorescens* on castor grey mold under four exposure times using detached capsule technique

plant roots. *P. fluorescens* induces plant systemic resistance, which reduces the pathogen infection (Abozaid et al., 2020). The siderophore producing *Pf* 34 and *Pf* 36 strains were most effective in inhibiting *B. ricini* on castor capsules at 12 hours exposure period.

4. Conclusion

Eight strains out of all the tested *P. fluorescens* were antagonistic to castor grey mold pathogen *B. ricini*. Among the chemicals, carbendazim showed maximum efficacy on the growth of *B. ricini* and in lowering capsule infection. The effectiveness of *P. fluorescens* strains *Pf* 34 and *Pf* 36 in inhibiting the growth of *B. ricini* on castor capsules at 12 hours exposure time has been clearly demonstrated and was on par with fungicide treated.

5. Further Research

Since the present study was conducted under laboratory conditions, more insight is required into the effect of exposure time of *B. ricini* spayed with bacterial (potential strains *P. fluorescens*) suspension alone or in combination with fungicides and non- conventional chemicals on disease infection under field conditions, more so because grey mold is a weather dependent disease. This would immensely help in taking prophylactic measures at appropriate time before the onset of the cyclonic rainfall and subsequently reduce the disease infection.

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