

Full Length Research Paper

***Penicillium citrinum* VFI-51 as biocontrol agent to control charcoal rot of sorghum (*Sorghum bicolor* (L.) Moench)**

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In our earlier investigation, a fungal isolate *Penicillium citrinum* VFI-51 and its secondary metabolite was reported to have antagonistic potential against *Botrytis cinerea*, the causative agent of *Botrytis* gray mold disease in chickpea. In the present investigation, *P. citrinum* VFI-51 was further evaluated for its antagonistic potential against *Macrophomina phaseolina*, the causative agent of charcoal rot in sorghum. *P. citrinum* VFI-51 inhibited *M. phaseolina* in both dual culture as well as secondary metabolite production assays. In the *in vivo* blotter paper assay, under light chamber conditions, *P. citrinum* VFI-51 controlled 85% of the charcoal rot disease on the roots when compared to the positive control. Under greenhouse conditions, when *M. phaseolina* was inoculated by tooth pick method in to the stalk of sorghum plant, the charcoal rot disease was controlled by 75% in *P. citrinum* VFI-51 treatment over the positive control. This study demonstrates the biocontrol potential of *P. citrinum* VFI-51 against charcoal rot of sorghum.

Key words: *Macrophomina phaseolina*, charcoal rot, sorghum, *Penicillium citrinum* VFI-51, biocontrol.

INTRODUCTION

Charcoal-rot of sorghum, caused by *Macrophomina phaseolina* (Tassi) Goid., is a root and stalk rot disease observed in most sorghum growing regions and endemic to tropical and temperate regions of the world (Wyllie, 1998). *M. phaseolina* is a soil borne pathogen causing

losses up to 64% in southern parts of India, in post rainy sorghum (Das et al., 2008). Improved high-yielding cultivars under good management practices also tend to be susceptible to the disease resulting in high yield losses (Mughogho and Pande, 1984). Symptoms of the

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charcoal rot disease includes premature drying of stalks, lodging of plants, soft stalks, root rot and poorly developed panicles with low quality grain formation. The most common indication is lodging of plants on reaching maturity (Uppal et al., 1936). A toxin called "phaseolinone" produced by *M. phaseolina* in the diseased stalk, causes anemia in mice (Bhattacharya et al., 1994). Though chemical control is available for the control of charcoal rot disease, the indiscriminate use of chemicals results in negative impact on nature (Rao et al., 2015). Further, the economic constraints of the small-scale farmers in semi-arid tropics limits them to use, chemical control (Gopalakrishnan et al., 2013).

Biological control can be the safe and alternative method to control this disease as it also contains plant growth-promotion (PGP) traits (Postma et al., 2003). PGP microbes control phytopathogens by producing different compounds such as siderophores, antibiotics, volatile compounds and a group of lytic enzymes such as chitinase, cellulase, lipase and protease (El-Tarabily et al., 2009). They also compete with the pathogen by inducing systemic resistance in plants (Compant et al., 2010). This group of microbes include bacteria such as *Pseudomonas* and *Bacillus*, actinomycetes such as *Streptomyces* and *Nocardia* and fungus such as *Trichoderma* and *Gliocladium* (Ding et al., 2004). The *Streptomyces* strains isolated from vermicompost were proved effective in controlling charcoal rot in sorghum and *Fusarium* wilt in chickpea (Gopalakrishnan et al., 2011a, b). In our previous study, we reported a strain of PGP fungus, *Penicillium citrinum* VFI-51, controlling *Botrytis* gray mold disease in chickpea caused by *Botrytis cinerea* (Sreevidya et al., 2015). In the present study, *P. citrinum* VFI-51 was tested for its ability to control charcoal rot of sorghum under both *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Microorganisms used in the study

A PGP fungus, reported earlier to have biocontrol potential against *Botrytis* gray mold disease in chickpea, *P. citrinum* VFI-51 (GenBank accession number: KM250379), was further studied in the present investigation for its antagonistic potential against charcoal rot in sorghum. The pathogen, *M. phaseolina*, was acquired from cereals pathology, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India.

In vitro dual culture and metabolite production assays

The fungus *P. citrinum* VFI-51 was screened for its antagonistic activity against *M. phaseolina* by dual culture assay as per the protocol of Gopalakrishnan et al. (2011b) on glucose casaminoacid yeast extract agar plates. Three replications were maintained for each treatment and control and, the experiment was repeated three

times. The plates were incubated at $28 \pm 2^\circ\text{C}$ for five days and zone of inhibition was recorded. For metabolite production assay, *P. citrinum* VFI-51 was grown in starch casein broth for five days at 28°C . At the end of incubation, the culture free filtrate of *P. citrinum* VFI-51 was collected and extracted by partitioning against ethyl acetate (EtOAc) and the resultant organic (EtOAc) and aqueous fractions were evaporated on a rotary evaporator and collected in a minimal volume of methanol. Both the fractions were evaluated for their antagonistic potential against *M. phaseolina*. For this, a fungal disc of 6 mm diameter of *M. phaseolina* was bored and kept at center of the potato dextrose agar plate amended with either organic or aqueous fractions (at a concentration of 0.5%). Control plates contained only 0.5% methanol. The plates were incubated at $28 \pm 2^\circ\text{C}$ for five days and growth of the pathogen was recorded.

In vivo blotter paper assay

Evaluation of *P. citrinum* VFI-51 for its efficacy against *M. phaseolina* was done by modified blotter paper method (Nene et al., 1981; Gopalakrishnan et al., 2011a) under light chamber conditions. The sorghum seeds susceptible to charcoal rot (variety R16) were surface sterilized with 2.5% sodium hypochlorite for two minutes and washed thoroughly with sterilized water. These seeds were sown in pots (12 cm) filled with sterilized vermiculite. The seedlings were collected after two weeks and the roots washed with sterilized water. The pathogen inoculum was prepared by growing *M. phaseolina* in potato dextrose broth (PDB) at $28 \pm 2^\circ\text{C}$ for five days and tissued using tissumizer (Techmar type T 25, Japan). For positive control, the roots of the sorghum seedlings were dipped in *M. phaseolina* inoculum for 30 min and arranged on blotter paper (45 x 25 cm with one fold) placed in a plastic tray, making sure only roots were present in the tray. For treatment, the roots of the sorghum seedlings were dipped in *M. phaseolina* inoculum for 30 min and arranged on blotter paper (45 x 25 cm with one fold) placed in a plastic tray and counter treated with *P. citrinum* VFI-51 (10^8 CFU/ml, 1 ml/plant; grown separately in PDB) to the sorghum roots. Ten plants were maintained per replication and three replications were maintained. Negative control was made by dipping the plants in sterile water. The blotter paper was kept moist all the time with sterilized water and incubated at $28 \pm 2^\circ\text{C}$ for eight days with a 12-h day length provided by fluorescent lights ($120 \mu\text{mol m}^{-2} \text{s}^{-1}$). At the end of the incubation, the rotting of roots that indicates disease symptoms of the charcoal-rot were recorded on a 0 to 5 rating scale (0 represents no visible disease symptoms, while 5 represents maximum disease symptoms), and the percentage of infected roots in treatments was calculated by comparing with the control.

Greenhouse study

Evaluation of *P. citrinum* VFI-51 for its efficacy against *M. phaseolina* under greenhouse conditions was done by tooth pick method (Edmunds, 1964). For this, pots (8") were filled with pot mixture containing black soil, sand and farm yard manure (3:2:1). Sorghum seeds (variety B 296) susceptible to charcoal rot were surface sterilized as mentioned earlier and soaked in *P. citrinum* VFI-51 grown in PDB. Three treated seeds were sown per pot but after germination only one plant per pot was maintained. A positive control, infected with *M. phaseolina*, and a negative control, without any inoculation, was also maintained. Each treatment contained 10 replications. Booster doses of *P. citrinum* VFI-51 were added on 0, 15, 30, 45 and 60 days after sowing by soil application.

For preparing the pathogen inoculum to infect the plant, the *M. phaseolina* was grown on PDA for five days at $28 \pm 2^\circ\text{C}$. The fungal

Table 1. Antagonistic activity of *P. citrinum* VFI-51 on *M. phaseolina* - in *in vitro* dual culture and metabolite production assays.

Treatment	Dual culture assay zone of inhibition (cm)	Metabolite production assay Fungal diameter (cm)
<i>P. citrinum</i> VFI-51	1.5	3.4
Control	-	9.0
SE±	0	0.06***

SE = Standard error; ***= statistically significant at 0.001.

Table 2. Antagonistic activity of *P. citrinum* VFI-51 on *M. phaseolina* - in *in vivo* blotter paper assay.

Treatment	Number of plants infected	% of roots infected	Visual rating
<i>P. citrinum</i> VFI-51	1.7	17	1
Control	10	100	5
SE±	2.4**		

*Mean of three replications; each replication contains 10 plants; visual rating of 0 to 5 rating scale (0 = no visible symptoms, while 5 represents maximum disease symptoms), SE = Standard error; **= statistically significant at 0.01

spores were scraped and transferred in to a sterilized honey peptone broth. Tooth picks were sterilized by keeping in a glass bottle; the above prepared fungal inoculum was poured in to this bottle up to one fourth of the bottle and incubated until the tooth picks were completely covered by the fungal growth. When the plants reach to flowering stage the plant was infected with the inoculated toothpick at second node from the ground level. After infecting, the plants were grown in stress and drought conditions, irrigation was given to maintain plant viability. At the time of harvesting, the above ground level stalks of the sorghum plants were collected and made transverse cut of the stalk to observe the length of infection and number of nodes infected.

Statistical analysis

Data were analyzed by using analysis of variance (ANOVA) technique, by SAS GLM (General Linear Model) procedure (SAS Inst. 2002-08, SAS V9.3).

RESULTS

In the present investigation, when *P. citrinum* VFI-51 was tested for its antagonistic activity against *M. phaseolina* under *in vitro* conditions, it inhibited *M. phaseolina* in both dual culture as well as secondary metabolite production assays effectively (Table 1). In the *in vivo* blotter paper assay, under light chamber conditions, *P. citrinum* VFI-51 controlled 85% of disease when compared to the positive control (Table 2 and Figure 1). Similarly, under greenhouse conditions, when *M. phaseolina* was inoculated by tooth pick method in to the stalk of sorghum plant, the charcoal rot disease was controlled by 75% over the positive control (Table 3 and Figure 2).

DISCUSSION

In our previous study, we reported the production of citrinin, a secondary metabolite, by *P. citrinum* VFI-51 which was responsible for controlling the *Botrytis* gray mold disease in chickpea. Production of citrinin was also reported by *Aspergillus* spp. and many species of *Penicillium*, including *P. citrinum* (Pitt, 2002). Citrinin is also reported for its antagonistic activity against soil and seed-borne plant pathogenic fungi such as *Sclerotium rolfsii*, *Rhizoctonia solani* and *Sclerotinia minor* (Melouk and Akem, 1987). In the present investigation, the organic fraction of the culture free extract of *P. citrinum* VFI-51 was found to inhibit *M. phaseolina* (Table 1) while in our previous study, the citrinin was extracted from the organic fraction only. Hence, it can be concluded that citrinin may be also responsible for the inhibition of *M. phaseolina*. Though, citrinin is a reported mycotoxin, it is non-phytotoxic and not altering ATPase activity, respiration and photosynthetic rates when applied on sorghum leaves (Damodaran et al., 1975). The LD₅₀ of citrinin on various animal models was also very high when compared with the concentrations used for the control of disease *Botrytis* gray mold (Sreevidya et al., 2015).

The control of charcoal rot disease in sorghum by *P. citrinum* VFI-51 could also be due to its capability to produce hydrolytic enzymes. In our previous investigation, *P. citrinum* VFI-51 was reported to produce siderophore, indole acetic acid (IAA), hydrocyanic acid (HCN), lipase, protease and β -1,3-glucanase. Siderophores help plants not only to acquire iron but also

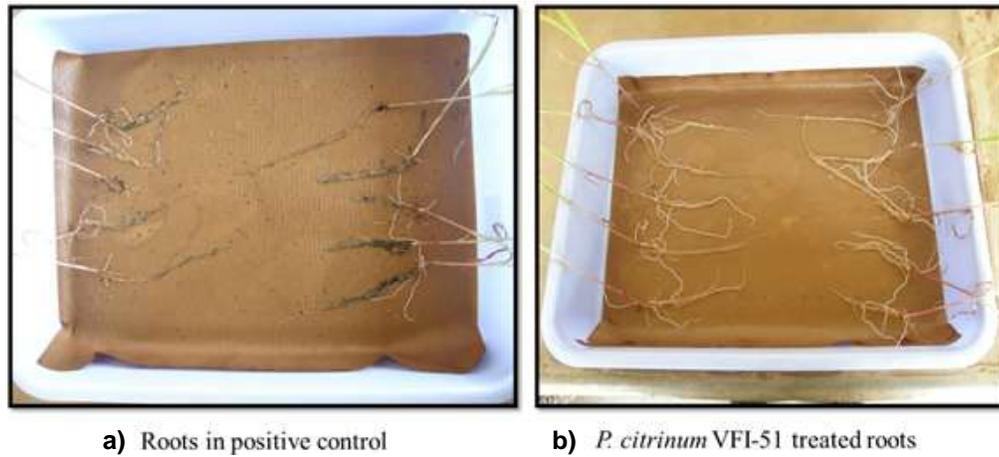


Figure 1. Antagonistic activity of *P. citrinum* VFI-51 on *M. phaseolina* - in *in vivo* blotter paper assay: (a) Positive control; (b) treatment.

Table 3. Antagonistic activity of *P. citrinum* VFI-51 on *M. phaseolina* - under greenhouse conditions

Treatment	Length of infection (cm)*	Infection %	Number of nodes infected*
<i>P. citrinum</i> VFI-51	2.4	25	1
Control	9.6	100	5
SE±	0.49***		

*= Mean of three replications; SE= Standard error; ***= statistically significant at 0.001.



Figure 2. Antagonistic activity of *P. citrinum* VFI-51 on *M. phaseolina* - under greenhouse conditions. (a) Negative control, (b) Positive control and (c) Treatment.

helps in disease suppression (Indiragandhi et al., 2008). IAA helps the host plants to stimulate seed germination, root formation and root elongation (Ahemad and Kibret, 2014) whereas HCN was also reported to help in disease suppression (Haas et al., 1991). Microorganisms producing lytic enzymes reported to play not only a role in nutrient mineralization and thus help the plants in growth promotion but also help in lysis of pathogenic fungal cell walls (Lima et al., 1998; Singh et al., 1999). The *Streptomyces* strains containing above mentioned biochemical properties were proved effective in controlling the soil-borne pathogens of chickpea and sorghum (Gopalakrishnan et al., 2011a, b). Khan et al. (2008) also reported production of gibberellins GA1, GA3, GA4 and GA7 by *P. citrinum* help in plant growth-promotion.

The *M. phaseolina*, causes the charcoal rot disease in sorghum when the plants are in stressed conditions such as high temperature and low moisture (Das et al., 2008). In our previous study, *P. citrinum* VFI-51 was also reported to tolerate harsh conditions such as high salinity (up to 20% NaCl), high pH (up to pH 11) and high temperatures (up to 40°C) and resistance to fungicides such as Bavistin and Thiram at field application levels (Sreevidya et al., 2015). Hence, *P. citrinum* VFI-51 can be exploited for controlling charcoal rot disease in sorghum.

From this study, it was confirmed that *P. citrinum* VFI-51 was able to control *M. phaseolina* under *in vitro* as well as *in vivo* conditions. Further studies needs to be carried out under on-station field conditions to prove efficacy of *P. citrinum* VFI-51 against charcoal rot disease. Further research also should be carried out to know the effect of citrinin in controlling the charcoal rot disease.

Conflict of Interests

The authors have not declared any conflict of interests.

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