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Eco-friendly management of *Colletotrichum gloeosporioides*, incitant of fruit rot of guava (*Psidium guajava)*

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ABSTRACT

The present study was conducted at Tej Narayan Banaili College, T.M. Bhagalpur University, Bhagalpur, Bihar during 2015-2017 to evaluate the efficacy of 8 medicinal plants viz. Datura stramonium (datura), Curcuma longa (turmeric), Azadirachta indica (neem), Zingiber officinale (ginger), Allium sativum (garlic), Ocimum sanctum (tulsi), Nyctanthus arbortristis (harsringar) and Piper betle (betel/paan) and 4 bioagents viz. Trichoderma harzianum, T. viride, Gliocladium virens and Chaetomium globosum against Colletotrichum gloeosporioides (Penz.) Penz. & Sacc., an incitant of fruit rot disease of guava (Psidium guajava Linn.). A maximum inhibition of mycelial growth of pathogen was recorded in garlic clove extract (80%) followed by betel leaf extract (76%), turmeric rhizome extract (74%), neem leaf extract (70%) and ginger rhizome extract (64%). Harsringar leaf extract was found as the least effective (56%). In pre-and post-inoculation tests, all the phytoextracts were found significantly superior in reducing Colletotrichum rot severity on 7 days after treatments as compared to control. Garlic clove extract again proved highly effective in reducing fruit rot severity after 7 days after inoculation in both pre - and post - inoculation treatments. In vitro dual culture experiments, maximum growth inhibition was recorded with T. viride (76.5%) followed by G. virens (72.2%), T. harzianum (67.6%) and least inhibition by C. globosum (44.7%) In both pre - and post- inoculation treatments with antagonists, maximum DRI (disease reduction index) was noticed with C. globosum (69.2 and 62.4%) followed by T. harzianum (51.9 and 56.7%), G. virens (44.8 and 46.9%) and least with T. viride (38.1 and 35.4%). The rot incidence at 8 DAI (days after inoculation) was significantly higher than that at 4 DAI. The hot water fruit dip treatment at 50^{O}C for 5 min was found best for controlling the rot without hampering the fruit quality.

Key words : Management, *Colletotrichum gloeosporioides*, fruit rot, guava

INTRODUCTION

Guava fruit suffers with a number of post-harvest rots caused by *Alternaria alternata*, *Aspergillus niger, Botryodiplodia theobromae, Colletotrichum gloeosporioides, Pestalotia psidii, Penicillium expansum* and *Rhizopus stolonifer* (Ray *et al.*, 2007; Singh *et al.*, 2008; Chourasia and Jha, 2010). Among various post-harvest rots of guava, fruit rot incited by *C. gloeosporioides* is a major disease in Bihar, having a pivotal role in guava cultivation with 29,000 ha area and annual production of 4,107 million tones in 2018 - 2019 (Anonymous, 2019). Under northern conditions, this disease appears in severe form during rainy season as compared to winter season crop. The genus *Colletotrichum* has occupied one of the top 10 fungal plant pathogens because of its broad host range, devastation of essential crops and importance as a model post-harvest pathogen (Munir *et al.*, 2016). *Colletotrichum* has been termed “a catalogue of confusion”, due to its perpetually changing taxonomy, limited morphological differentiation among species, rare presence of sexual stages and variation in pathogenicity and cultural morphology (Cannon *et al.,* 2012; Johnson, 2018). *Colletotrichum* spp. vary in their infection strategies on different hosts and species, and may preferentially infect different tissues (De Silva *et al.*, 2017). This soil borne pathogen (*C. gloeosporioides*) may produce toxic metabolites whose ingestion by human beings may lead to several disorders known as mycotoxicoses (Li *et al.*, 2017). Although various fungicides have shown promosing results in controlling post-harvest fruit rot pathogens of guava, but the development of fungicidal resistance in pathogens, contamination of food by toxic residues, resurgence of pest and detrimental effects on non-target organisms are some of the major constraints in disease management. Biological control has emerged as a potential strategy to combat major post-harvest fruit rot pathogens.

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Successful biological control systems commonly employ naturally occurring antagonistic fungi and bacteria that are effectively reduce activities of plant pathogens (Cook, 1993;Shishido *et al.*, 2005). *Trichoderma* spp. are the filamentous soil borne mycoparasitic fungi commonly used as bio-fungicides for the management of a wide range of soil borne plant pathogens and some have a plant growth promotion ability (Tasiwal *et al.*, 2009; Ozbay *et al.*, 2014). During the last few decades they have enabled non-chemical plant disease management system and organic agriculture in particular (Woo, 2006; Pan and Bhagat, 2007). Several plant species have also shown inhibitory activities against fruit rot pathogens (Lakpale *et al.*, 2008; Panchal and Patil, 2009). In the present investigation, some eco-friendly methods were tried for the management of *Colletotrichum gloeosporioides* fruit rot of guava with some phytoextracts, biocontrol agents especially *Trichoderma harzianum, T. viride, Gliocladium virens* and *Chaetomium globosum,* and hot water treatment.

MATERIALS AND METHODS

Pathogenicity test and identification of incited rot:

The pathogencity was proved by inoculating the injured healthy fruits with spore suspension ($1x10^6$ spores/ml) of *Colletotrichum gloeosporioides* isolated from rotted guava fruits, collected randomly from retail fruit markets and cold storages of Bhagalpur during 2015 - 2017. Healthy fruits with pedicels were injured several times with the help of a fine sterilized needle. A cotton swab soaked with spore suspension was placed on the injured area. Fruits were then covered with perforated polythene bags and incubated at $25\pm1^{O}C$ for 7 days. The cotton swab was moistened with sterile distilled water whenever needed. Typical symptoms of the rot were recorded after 7 days. The identification of *C. gloeosporioides* was done with the help of identification key of imperfect fungi (Barnett and Hunter, 1972).

*Efficacy of phytoextracts***:**

Eight medicinal plants viz., datura (*Datura stramonium* L), turmeric (*Curcuma longa* L.), neem (*Azadirachta indica* A. Juss), ginger (*Zingiber officinale* Rose), garlic (*Allium sativum* L.), tulsi (*Ocimum sanctum* L.), harsringar (*Nyctanthes arbortristis* L.) and betel (*Piper betle* L.) were evaluated against *C. gloeosporioides* by following poisoned food technique (Nene and Thapliyal, 1979). Each of the phytoextracts was thoroughly mixed in sterilized 100 ml PDA medium under aseptic condition. The medium was supplemented with streptomycin sulphate @ 50 ppm to prevent bacterial contamination. The plates were inoculated with a 5 mm disc of 7 days old culture of test pathogen along with suitable control. Each treatment was replicated four times. The inoculated plates were incubated at $28\pm1^{O}C$. Observations on growth inhibition and sporulation of test fungi were recorded after 7 days of incubation (Panchal and Patil, 2009). The sporulation was recorded in a l - 5 scale: 1 = no spore, 2 = poor (1 - 7), 3 = moderate (8 - 25), 4 = good (26 - 50) and 5 = excellent (>50 conidia per microscopic field).

*Pre-inoculation method***:**

The healthy, semi-ripe, uniform size guava fruits were surface sterilized by dipping in $HgCl_2$ (1%) for 1 min followed by three washings with sterile distilled water. The spores from 7 days old cuture of *C. gloeosporioides* were scrapped with the sterile needle and mixed in 200 ml distilled sterile water. The fruits were first dipped in phytoextracts (10%) separately and then inoculated after 12 hr with the pathogen. The fruits were injured with sterilized cork borer at stem-end and dipped in spore suspension (10^6 spores/ml) for 2 min and air dried for 15-20 min. The inoculated and un-inoculated fruits were placed individually in sterilized polythene bags. A piece of sterilized moist absorbent cotton swab was placed inside the bag and mouth of the bag was loosely tied. The bagged fruits were incubated at $28\pm1^{O}C$ for 7 days. Each treatment was replicated eight times.

Post-inoculation method:

In post-inoculation treatment, the semi-ripe fruits were first inoculated with the pathogen and then treated with the phytoextracts. Further procedure was followed as mentioned earlier. In vitro evaluation of antagonists**:** Efficacy of four fungal antagonists viz. *Trichoderma harzianum, T. viride, Gliocladium virens* and *Chaetomium globosum* were tested by dual culture technique against *C. gloeosporioides.* Mycelial discs of 5

mm diam cut from margin of 7 days old culture of the pathogen and fungal biocontrol agents were placed 6 cm apart on PDA (potato dextrose agar) in the same Petri plate opposite to each other. Plates having only pathogen served as control. Each treatment was replicated thrice. The inoculated plates were incubated at 25±1^{O}C in a BOD chamber. The per cent inhibition in growth of pathogen by biocontrol agents over control was calculated as Jat *et al.* (2008).

*Non-volatile metabolites***:**

Hundred ml sterilized PDB (potato dextrose broth) filled in 250 ml flasks incubated at 25±1^{O}C for 15 days in a BOD chamber. The broth was filtered through three layered Whatman filter paper No. 42. Each filtrate was mixed with PDA separately (10%) and autoclaved in flasks. The PDA was then poured aseptically into sterile Petri plates. After solidifying the PDA, the mycelial discs of 5 mm diam were cut from the margin of 7 days old actively growing culture of the pathogen and inoculated in the centre of the plates. Plates having only PDA without filtrate served as control. Each treatment was replicated thrice. The inoculated plates were incubated at 25±1^{O}C in a BOD chamber. The per cent inhibition in mycelial growth of pathogen over control was derived.

*Volatile metabolites***:**

The efficacy of volatile metabolites was studied by inoculation of the biocontrol agents and the pathogen with 5 mm disc of 7days old actively growing culture in separate Petri plates on PDA. Lids of Petri plates were removed and the Petri plate containing pathogen was inverted over the Petri plate containing biocontrol agent and sealed with adhesive tape (magic tape) under aseptic conditions. The control had same pathogen in both upper and bottom Petri plates. Inoculated Petri plates in triplicate were incubated at 25±1^{O}C in a BOD chamber. The per cent inhibition in mycelial growth of the pathogen as compared to control was calculated.

*In vivo evaluation of antagonists***:**

Antagonists studied *in vitro* were further tested with guava fruits. The healthy semi-ripe fruits were surface sterilized by dipping in $HgCl_2$ solution (0.1%) for 1 min followed by three washing with sterile distilled water and pricked up to the depth of 2 mm, making 5 wounds/fruit. These fruits were separately inoculated by dipping them in a spore suspension (10^6 spores/ml) for 2 min. Spore suspension of each biocontrol agent was used as pre - as well as post - inoculation dip treatments. In pre-inoculation treatment, the fruits were first dipped in the spore suspension of biocontrol agents for 5 min, air dried for 15 min and then inoculated with pathogen. In the post-inoculation treatment, the fruits were first inoculated with pathogen and then treated with biocontrol agents. Parallel controls with fruits dipped in sterile distilled water were run simultaneously. The interval between inoculations was of 12 hr. Each treatment was replicated thrice with 7 fruits per replication in a factorial randomized block design. The inoculated fruits were enclosed separately in pre- sterilized perforated polythene bags partially sealed with paper pins and incubated at 25±1^{O}C and 90-100% RH. The number of wounds showing rot were recorded on 4 and 8 DAI (day after inoculation) and per cent rot incidence was calculated. The disease reduction index (DRI) was worked out as Sharma *et al.* (2008).

*Hot water treatment***:** The effect of hot water treatment on the development of *C. gloeosporioides* fruit rot was studied at 45, 50 and 55^{O}C. The semi-ripe guava fruits were inoculated with pathogen (10^6 spores/ml) by stem end injury method and exposed to desired temp for 5 and 10 min along with control with a 12 hr interval between inoculation and hot water treatment. All the fruits were placed separately in sterilized polythene bags along with sterilized moist cotton swab and bagged fruits were incubated at 28±1^{O}C. Each treatment was replicated eight times. Observations on the disease severity were recorded after 7 days of inoculation.

RESULTS AND DISCUSSION

Identification of rot symptom, pathogen and pathogenicity test

The rot symptom on guava fruit was identified by the appearance of whitish cottony growth which developed very fast as the fruit matures and pathogen soon covered the entire

surface within a period of 3-4 days under humid weather. The skin of the fruit below the whitish cottony growth become soft and turned light brown to dark colour. The rot gradually extended deep into the fruit rendering the tissues black, soft and watery. The repeated isolation of *C. gloeosporioides* from rotten portions of the fruits proved pathogenicity in the test.

Phytoextracts

All the phytoextracts were found significantly superior in inhibiting the mycelial growth and sporulation of *C. gloeosporioides* over control (Table 1). A maximum inhibition was recorded in garlic clove extract (10 mm) with 80% inhibition over control. The next best treatments were obtained with betel (paan) leaf extract (13 mm), turmeric rhizome extract (15 mm), neem leaf extract (17 mm) and ginger rhizome extract (20 mm) with 76%, 74%, 70% and 64% inhibition, respectively. Harsringar leaf extract was found as the least (56%) effective. None of the phytoextracts supported sporulation which was abundant in control. Similar results on inhibition of *C. gloeosporioides* by betel leaf extract have been reported by Johnny *et al.* (2010). They stated that the antifungal properties of betel is due to the presence of a number of physiologically active compounds such as alkaloids / amides, propenylphenols, lignans, neolignans, terpenes, steroids, kawapyrones, piperolides, chalcones, flavones and flavanones. Jat *et al.* (2008) also found neem leaf extract as effective in inhibiting the mycelial growth of *C. gloeosporioides* causing banana fruit rot. Singh (2011) reported that the neem extract contains a high level of antifungal azadirachtin compound. Other researchers have reported similar results with different fruits using garlic clove extract (Shinde *et al.*, 2016; Nurfatimma *et al.*, 2018). According to Alemu *et al.* (2014), allicin, dially trisulphide and ajoene are the active compounds in garlic that give inhibitory activity against *C. gloeosporioides* in mango anthracnose. Lakpale *et al.* (2008) and Dissanayake *et al.* (2019) reported that extracts of several botanicals were highly effective in inhibiting mycelial growth and sclerotial production of different soil borne plant pathogens.

Table 1: Effect of phytoextracts (10%) on mycelial growth of *C. gloeosporioides* and severity (%) of guava fruit rot in pre-and post- inoculation tests

Phytoextracts	Mycelial growth (mm)	Growth inhibition (%)	Pre-inoculation	Post-inoculation
Betel (Paan) leaf extract	13	76	10	14
Datura leaf extract	21	62	12	16
Garlic clove extract	10	80	7	11
Ginger rhizome extract	20	64	16	21
Harsringar leaf extract	27	56	17	25
Neem leaf extract	17	70	14	18
Tulsi leaf extract	22	61	14	17
Turmeric rhizome extract	15	74	22	28
Control	58	0	42	39
CD (P=0.05)	2.95		2.76	3.35
CV (%)	8.25		7.95	8.25

**Figures were square root transformed before analysis*

In pre- and post-inoculation tests, all the phytoextracts were found significantly superior in reducing the *Colletotrichum* rot severity on 7 days after treatment as compared to control (Table 1). In pre-inoculation treatment, a reduced rot severity was observed in guava fruits treated with garlic clove extract followed by betel, neem and tulsi leaf extracts. In post-inoculation method, significant reduction in fruit rot severity was found with extracts of garlic, betel, datura, tulsi and neem. Extracts of turmeric rhizome and harsringar had least effect on development of fruit rot symptoms in both pre- and post - inoculation treatments.

Efficacy of bioagents

All the 4 biocontrol agents screened under dual culture experiments inhibited mycelial growth of *C. gloeosporioides* significantly over

control. A maximum inhibition of growth (76.5%) was recorded with *T. viride* followed by *G. virens* (72.2%), *T. harzianum* (67.6%) and least inhibition (44.7%) by *C. globosum* (Table 2). *T. viride* and *G. virens* were statistically at per with each other in limiting *C. gloeosporioides.*

Table 2: *In vitro* antagonism (% inhibition) by fungal biocontrol agents against *C. gloeosporioides*

Biocontrol agents	Dual culture*	Volatiles	Non-volatiles
Trichoderma harzianum	67.6	58.6	49.6
T. viride	76.5	64.2	57.7
Gliocladium virens	72.2	62.4	54.8
Chaetomium globosum	44.7	25.6	32.4
Control	0	0	0
CD (P=0.05)	2.27	2.35	2.3
CV (%)	2.77	3.45	3.20

**Figures were square root transformed before analysis*

Results of pre-inoculation experiments with antagonists were found significantly efficient in reducing fruit decay loss over control (Table 3). *Chaetomium globosum* retarded (69.2%) the rot incidence most effectively. The next best antagonist was *T. harzianum* (51.9%). *G. virens* checked the rot incidence moderately (44.8%). The least disease reduction was provided by *T. viride* (38.1%). As the incubation period increased, the severity of rot incidence was also increased. In post- inoculation treatment, all biocontrol agents tested suppressed the rot incidence in guava fruits significantly as compared to control (Table 3).

Table 3: Effect of biocontrol agents on incidence (%) of post-harvest fruit rot in guava

Biocontrol agents	Pre-inoculation*			Post-inoculation*		
	4 DAI	8 DAI	DRI	4 DAI	8 DAI	DRI
Trichoderma harzianum	25.6	37.4	51.9	30.5	42.7	56.7
T. viride	30.2	46.8	38.1	34.6	58.4	35.4
Gliocladium virens	27.0	42.5	44.8	31.2	46.3	46.9
Chaetomium globosum	16.1	25.8	69.2	19.1	32.7	62.4
Control	54.7	77.2	0	55.5	85.2	0
		CD (P=0.05)				
Biocontrol agents		1.44			1.72	
Days		0.71			0.92	
Interaction		2.10			2.42	
CV (%)		3.37			3.78	

**Figures were angular transformed before analysis; DAI = days after inoculation; DRI=disease reduction index*

The results on dual culture with biocontrol agents revealed that out of 4 antagonists, *T. viride* proved most inhibitory followed by *G. virens* towards mycelial growth by *C. gloeosporioides*. Similar observations on biocontrol potential of *Trichoderma* species and other fungi against fruit rot pathogens have been reported by various workers (Sharma *et al.*, 2008; Joshi *et al.*, 2016). Results similar to the present investigation were reported by Cruz-Quiroz *et al.* (2018) showing significant mycelial growth inhibition of *C. gloeosporioides* and *Phytophthora capsici* by native Mexican *Trichoderma* strains. Further Valenzuele *et al.* (2015) and Maheshwari and Vidhya (2016) found *T. viride* and *T. harzianum* as the most potent bio-agents in inhibiting mycelial growth of various fruit rot pathogens. The use of *T. viride* and *T. harzianum* significantly checked the *Fusarium* wilt of cumin and chickpea under greenhouse and field conditions (Gangopadhyay and Ram Gopal, 2010; Khan and Gangopadhyay, 2012).

Various modes of action of the biocontrol agents in suppressing the soil borne pathogens including *Alternaria alternata*, *C. gloeosporioides*, *Fusarium* species have been demonstrated. For instance, the mode of antagonism of *Trichoderma* and *Gliocladium* spp. to pathogens are known to be through competition for nutrition and space, and production of lytic enzymes, toxins and volatile substances (Agarwal *et al.*, 2011; Khan and Gangopadhyay, 2012; Mukhopadhyay and Kumar*, 2020*). *Trichoderma* spp. are known to produce volatidle (6-pentyl α- pyrone and harzionalide) and non-volatile (trichodermin, suzukacillin and alamethicine) antibiotics (Joshi *et al.*, 2016). In the present investigation, secreation of yellowish green or light brown secondary metabolites by *Trichoderma* spp. in growth medium was observed. Based on scanning electron microscopical investigation, Kishan *et al.* (2017) put the evidences of mycoparasitism of *Sclerotinia sclerotiorum* by *Trichoderma* spp.

Hot water treatment

All the hot water treatments were found significantly superior in reducing the guava fruit rot severity on 7 days of inoculation over control (Table 4). The fruits exposed to 55^{O}C for 10 min recorded significantly lowest *Colletotrichum* fruit rot but it was at par to 55^{O}C for 5 min or 50^{O}C for 10 min. The natural skin colour of fruits changed from yellowish white to dark brown with softening of underlying tissue due to high temperature. Soares-Colletti and Lourenco (2014) reported that a hot water dip of guava fruits at 50^{O}C for 2 min followed by irradiation at 0.5 KGy totally eliminated fruit decay loss caused by *Colletotrichum* spp.

Table 4. Effect of hot water treatment on the severity of *C. gloeosporioides*

Hot water treatment	% severity after 7 days*
45^{O}C for 5 min	16
45^{O}C for 10 min	10
50^{O}C for 5 min	08
50^{O}C for 10 min	05
55^{O}C for 5 min	04
55^{O}C for 10 min	04
Control	37
CD (P=0.05)	2.6
CV (%)	18.4

**Figures were square root transformed before analysis*

The present study concluded that the post-harvest fruit decay loss of guava could be controlled by using extracts of garlic clove, betel leaf, turmeric rhizome and neem leaf; biocontrol agents viz. *T. viride, T. harzianum*, *C. globosum* and *G. virens*, and hot water treatment at 50^{O}C for 5 min. In order to prevent further advancement of diseae to a damaging level, this biocontrol consortium may be ideal to use.

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