Effects of Biocontrol Agents and Plant Products on *Macrophomina phaseolina* and Colchicine Content in *Gloriosa superba*

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Abstract

Alice D., Sundravadana S. (2012): **Effects of biocontrol agents and plant products** *Macrophomina phaseolina* **and colchicine content in** *Gloriosa superba*. Plant Protect. Sci., **48**: 110–115.

Gloriosa superba is a medicinal plant severely infested with soil-borne *Macrophomina phaseolina* (Tassi) Goid. Under *in vitro* conditions a commercial formulation of *Trichoderma viride* and *Pseudomonas fluorescens* inhibited the mycelial growth of *M. phaseolina* isolates. Among the oil cake, mahua cake at 10% completely inhibited the mycelial growth of the *M. phaseolina* isolates. Under field conditions both the soil and foliar application of biocontrol agents is attributed to the healthy growth of *G. superba* crops by controlling the tuber rot disease and ultimately boosting the colchicine content.

Keywords: Macrophomina blight; tuber rot; *Trichoderma*; *Pseudomonas*; oil; soil application; foliar application

Gloriosa superba L. is a medicinal plant belonging to the family *Liliaceae*. It occurs naturally in Africa, India, Southeastern Asia and is nowadays distributed widely throughout the tropics, and worldwide as a pot plant. The tuber is claimed to have antidotal properties to snakebites. In the Indian systems of medicine, the tubers are used as tonic, antiperiodic, anthelminthic, and also against snakebites (Gupta *et al*. 2005). The tuber of *G. superba* is a rich source of colchicine, which has shown anti-gout, anti-inflammatory, and antitumor activity (Pandey *et al.* 2008).

In India, the Tamil Nadu state leads in the production of glory lily with annual production of 800 t of dry seeds. Its cultivation has extended up to 2500 ha with major regions comprising Tirupur, Erode, Dindugal, Karur, Ariyalur, and Nagapattinam districts. The annual foreign exchange through the export of glory lily seeds is estimated to be Rs.100 crores. Tubers are an important source as planting material and of colchicine. Even though better returns are expected from this high-value crop, it is severely infested with the soil-borne fungal pathogen *M. phaseolina*, tubers are rotted and cause 25% to 30% losses both in field and storage conditions. If the propagating material is subjected to tuber rot, the tuber will fail to sprout since the vigour of the vine and its flowering and fruiting ability depend on disease-free tubers.

M. phaseolina (Tassi) Goid., a soil-inhabiting fungus, is an important root pathogen and causes dry root rot/stem canker, stalk rot or charcoal rot of over 400 plant species including *G. superba*. Since *M. phaseolina* is a soil-borne fungus, it poses a greater problem in managing the disease. Soil-borne diseases are difficult to control. The use of antagonistic organisms against *Macrophomina* root rot has been well documented in several crops (Lokesha & Benagi 2007; Anis *et al.* 2010). Sundravadana (2002) reported that the seed and soil application of *T. viride* significantly controlled the blackgram root rot caused by *M. phaseolina.*

Hence, the aim of this study was to investigate the antifungal activity of biocontrol agents and

plant products on *M. phaseolina* and its controlling activity against tuber rot incidence and colchicine concentrations of *G. superba*.

MATERIAL AND METHODS

***Source of** M. phaseolina **isolates**.* Survey and tuber sampling were done randomly from individual plants of *G. superba* that were naturally infected by *M. phaseolina*. Samples were collected in different places in the Tamil Nadu state, India, and transported in plastic bags to the laboratory and then either immediately used for the pathogen isolation or stored in a refrigerator for subsequent usage. Segments of *G. superba* tubers were sterilised (0.1% mercuric chloride) and washed with distilled water three times, then transferred into a 90 mm Petri plate with PDA medium. The isolates were incubated at 25°C to 30°C, which allowed them to grow, then they were transferred into PDA slants.

In vitro assay of responses to biocontrol agent. *T. viride* isolates were isolated from the soil using a *Trichoderma* selective medium (TSM) developed by Elad and Chet (1983) following the dilution plate technique. The inhibitory effect of *T. viride* isolates against *M. phaseolina* isolates was determined by the dual culture technique. The fungal antagonists and the pathogen were cultured on PDA medium. the test antagonist (8 mm) were then cut from the periphery of the colony and placed at one end of the sterilised Petri dish containing 15 ml of PDA medium. A similar disc of the pathogen was placed at the opposite end approximately 75 mm from the first. The linear growth of the antagonist and the pathogen was measured at regular intervals after inoculation.

In vitro assay of responses to plant products. The inhibitory effect of the plant oils (0.1%) from neem, mahua, pungam, castor and oil cake (10%) from mahua, groundnut, coconut, neem and cotton on *M. phaseolina* isolates was determined using an *in vitro* test on amended agar plates. The control plate contained only agar. There were three replicate plates for each of the tested isolate. A 9-mm mycelial plug was cut using a cork borer from the margin of the colonies of *M. phaseolina* and transferred into the middle of 9-cm Petri dishes containing amended agar. After 3 days incubation at 28 ± 2°C, the colony diameters in the dishes were measured on all plates when the mycelial growth of the control reached the edge of the plate.

A difference in colony diameter between the poisoned medium and the control was used to calculate the per cent inhibition as follows:

$$Mi = \frac{Mc - Mt}{Mc} \times 100$$

where

Mi – inhibition of mycelial growth

Mc – colony diameter of the control set

Mt – colony diameter of fungi on the poisoned medium

Efficacy of biocontrol formulations and plant extract in the control of tuber rot of glory lily under storage

Trichoderma viride formulation. Talc-based formulation of *Trichoderma* was prepared according to the method described by Jeyarajan et al. (1994). Eight mm disc of 5 days old culture grown on PDA was inoculated in 250 ml of conical flasks containing 100 ml of yeast molasses medium. Eight days after incubation, the contents of the flask including the mycelial mat and metabolite were homogenized. Later it was mixed with talc powder at the ratio of 1:2 (v/w) and shade dried for two days. About 10 g of Carboxy Methyl Cellulose (CMC) was added as sticking material per 1 kg of talc powder.

Pseudomonas fluorescens formulation. *Pseudomonas* was grown in Kings B broth for 48 h as shake culture incubating in an Infors AG shaker at 150 rpm at room temperature (28 ± 2°C). CMC (10 g) was added to 1 kg of talc and mixed well. The pH of the media was adjusted to 7.0 by adding calcium carbonate. The talc was autoclaved for two consecutive days for about 30 minutes. Four hundred millilitres of the bacterial suspension containing 9×10^8 CFU/ml were added to 1 kg of the carrier and mixed well under sterile conditions. The materials were packed in a polythene bag, sealed and incubated at room temperature (28 ± 2°C) (Vidhyasekaran & Muthamilan 1995).

The tubers of G. *superba* are bulked under a thatched shed before sowing. During storage the tubers are severely infected by *M. phaseolina* causing tuber rot. Hence to prevent the storage loss of seed tubers, the enriched composted coirpith [1 t of coirpith sandwiched with mushroom culture (*Pleurotus sajor-caju)* 500 g, 5 kg urea and 5 kg zinc sulphate and the heap was sprinkled with 1% cow dung slurry for early composting] was mixed

separately with *T. viride* 1 kg and *P. fluorescens* 1 kg. For 100 tubers 5 kg of the above composted materials were spread at the base and 5 kg were spread at the top. The control was as that of the farmers' practice, i.e. bulked tubers. Two field trials were conducted in a G. *superba* growing area of Tamil Nadu, where the disease occurs annually.

Efficacy of biocontrol formulations and plant extract in the control of tuber rot of glory lily under field conditions. Two field trials were conducted in the glory lily growing area of Tamil Nadu, where the disease occurs annually. The talc-based powder product of biocontrol agents (2.5 kg/ha) was applied basally as soil application. For foliar application, the talc-based formulation was thoroughly mixed in water (20 g/l), allowed to settle for 1 h, filtered through muslin cloth and the filtrate was sprayed at 45 days and 90 days after sowing (DAS). An untreated control was also maintained. The trials were laid out in a randomised block design (RBD) with three replications. The incidence of tuber rot was recorded 120 days after sowing.

Estimation of colchicine content in seed – HPLC analysis. The colchicine content (in %) in leaves, tubers, seed, seed coat and husks was estimated on the principle that colchicine on acid hydrolysis yields colchicine which in reaction with ferric chloride ($FeCl_3$) records an olive green colour. The intensity of green colour was read in a spectrophotometer at 470 nm.

Table 1. Effect of biocontrol agents on *M. phaseolina* (*in vitro*)

Isolates *M. phaseolina*	*Trichoderma* species*											
	mycelial growth (mm)	inhibition over control (%)	mycelial growth (mm)	inhibition over control (%)	mycelial growth (mm)	inhibition over control (%)	mycelial growth (mm)	inhibition over control (%)	mycelial growth (mm)	Inhibition over control (%)	mycelial growth (mm)	inhibition over control (%)
	T. viride commercial formulation		*T. viride* NI-1		*T. viride* NI-2		*T. viride* NI-3		*T. viride* NI-4		*T. viride* NI-5	
I_1	30.1	67	58.20	34	70.30	20	78.50	11	85.00	4	68.40	22
I_2	0.0	100	58.60	34	71.20	19	79.50	10	85.30	4	67.20	24
I_3	48.2	46	66.20	25	74.20	16	80.40	9	84.90	4	65.30	26
I_4	70.2	22	63.20	29	69.50	21	80.70	8	84.70	5	67.80	23
I_5	38.2	58	61.00	31	75.10	15	82.30	6	85.60	4	68.20	23
I_6	72.0	39	69.70	21	75.40	14	81.10	8	86.70	2.	78.50	11
I_7	15.1	83	60.40	32	70.00	20	80.00	9	87.50	1	80.00	9
Mean	45.2		65.91		74.20		81.31		85.96		72.92	
Control	88.0		90.00		88.00		88.00		88.00		88.00	
CD	5.34		4.34		5.12		7.24		2.99		4.46	
	T. viride NI-6		*T. viride* NI-7		*T. viride* NI-8		*T. viride* NI-9		*T. viride* NI-10		*P.fluorescens*	
I_1	77.30	14	79.80	11	84.20	4	79.90	9	77.80	12	23.10	74
I_2	79.90	11	88.60	2	85.60	3	78.50	11	78.60	11	32.10	64
I_3	86.50	4	84.90	6	88.00	0	86.30	2	82.90	15	32.50	63
I_4	78.90	12	85.60	5	85.60	6	85.20	3	75.60	14	30.40	65
I_5	82.50	8	88.30	2	84.30	4	86.30	2	78.30	11	45.80	48
I_6	88.30	1	84.60	6	84.20	4	87.20	1	83.60	5	45.20	49
I_7	84.60	6	85.90	5	82.60	2	86.00	2	74.90	6	10.00	89
Mean	90.00		8.70		88.00		88.00		88.80		88.00	
Control	83.50		75.80		85.31		84.67		80.06		38.38	
CD	4.58		5.28		4.58		4.58		5.28		5.28	

*mean of three replications

The dried and powdered material (1 g) was added 100 ml of 1 percent hydrochloric acid and stirred for one hour. It was kept in a water bath for half an hour at 50°C. After cooling to room temperature it was left for freezing. Then it was filtered through Whatman No. 1 filter paper and the filtrate was collected in a 100-ml conical flask. From the flask 10 ml of the filtrate was taken in a test tube and 0.1 ml of 5% ferric chloride solution was added and the OD value was read at 470 nm. The standard colchicine for the analysis was obtained from M/S Sigma Chemicals (St. Louis, USA). The linear graph was drawn by finding out OD values (absorbency) for concentrations ranging from 100 ppm to 1000 ppm. The concentration (ppm) corresponding to the OD value of the sample was determined from the graph and colchicine content was calculated as given below using the following formulae in terms of ppm after which it was converted into per cent.

$$\text{Sample quantity (ppm)} = (A_s / A_{std}) \times (M/M_1) \times (V/V_1) \times F$$

where:

A_s – sample area
A_{std} – standard area
M – standard injected (µl)
M_1 – weight of the sample taken
V – volume of the final extract
V_1 – sample injected (µl)
F – recovery factor

Colchicine content = (Concentration (ppm) from the graph × 100)/(1 × 10 000)

Colchicine content was estimated in seed in each treatment and expressed in %.

RESULTS AND DISCUSSION

The survey was conducted in *G. superba* extensive growing areas of Tamil Nadu, viz. Mettupalayam, Ranganvalasu, Ambligai, Chinnagoundan valasu, and Moolanur. The survey revealed that tuber rot caused by *M. phaseolina* was prevalent and the infection ranged from 20% to100 %. The fields in which poultry manure and farmyard manure were applied recorded 100% incidence of tuber rot. The tuber infection was surveyed before sprouting and the infection starts from the tips or in the centre of the tuber. Mycelial growth was seen in the tubers.

Among the seven isolates of *M. phaseolina* I_2 was completely inhibited by the commercial formulation of *T. viride.* The other isolate inhibition ranged from 22% to 83% (Table 1). The other native isolates (1–10) were not effective against the seven isolates of *M. phaseolina.* Commercial formulation of the bacterial antagonist *P. fluorescens* was tested against *M. phaseolina* isolates under *in vitro* conditions. Among the seven isolates of *M. phaseolina* I_7 recorded 88.63 % inhibition over the control against *P. fluorescens.* The other isolate inhibition ranged from 47.95% to 73.75%.

Plant oils (0.1%), viz. Neem, Pungam and castor, were highly inhibitory to the mycelial growth of *M. phaseolina* isolates. Among the oil cakes, mahua

Table 2. Effect of plant oil and oil cakes on the mycelial growth (mm) of *M. phaseolina in vitro*

M. phaseolina isolates	Oil			Cake				
	Neem	Pungam	castor	Neem	mahua	cotton	groundnut	coconut
I_1	4.33	1.00	0.67	85.6	0.0	86.7	87.4	88.2
I_2	7.00	3.33	2.00	87.2	0.0	85.4	87.2	88.3
I_3	3.33	4.00	2.33	85.9	0.0	85.3	86.9	87.9
I_4	5.33	3.67	1.00	84.5	0.0	86.2	86.5	87.5
I_5	5.00	4.00	2.33	87.6	0.0	86.4	87.2	86.9
I_6	3.70	3.33	1.67	83.4	0.0	82.1	82.1	88.5
I_7	3.00	2.67	1.00	85.9	0.0	88.0	86.7	85.6
Mean	89.67	89.33	89.67	90.0	0.0	88.00	88.0	89.0
Control	89.00	89.33	89.67	90.0	89.0	88.00	88.0	79.0
CD	0.96	0.94	0.95	4.39		4.58	5.92	4.42

*mean of three replications

Table 3. Management of tuber rot of *Gloriosa superba* (field trials 2009–2010)

Treatments		Macrophomina blight		Tuber rot	
		disease (%)	reduction of disease (%)	disease (%)	reduction of disease (%)
T_1	Mahua oil 10% (two sprays)	25	16	15	53
T_2	Basal soil application of *T. viride* @ 2.5 kg/ha and spray with *T. viride* 0.2%	12	58	13	61
T_3	Basal soil application with *P. fluorescens* @ 2.5 kg/ha and spray with *P. fluorescens* 0.2%	13	59	13	61
T_4	Basal application of *P. fluorescens* and *T. viride* spray with 0.5% zinc sulphate	12	60	11	67
T_5	Basal application of iluppai cake @150 kg/ha and spray with iluppai oil 3%	15	50	12	63
T_6	Carbendazim 0.1% drenching (45 days) and one foliar spray (90 days)	11	66	8	75
T_7	Control	30	0.0	32	0.0
	CD (0.05)	1.94		1.29	

For all the treatments except the control the basal application of zinc sulphate @ 25 kg/ha was done
All the treatments were carried out twice, the first at 45 days after germination and the second at 90 days after germination

cake at 10 % was completely inhibiting the mycelial growth of the Macrophomina isolates. The other oil cakes (10%) viz., Neem, cotton, groundnut and coconut were ineffective (Table 2).

Controlling activity of biocontrol agent and plant products against tuber rot diseases

The tubers of G. *superba* are bulked under thatched sheds before sowing. They were severely infected by *M. phaseolina* (latent infections). The results revealed that the composted coirpith with zinc sulphate used for sandwiching the tubers recorded only 10% infection whereas the control (farmers' practice) recorded 35% infection (data not shown).

Among the various treatments the basal application of *T. viride* (soil application @ 2.5 kg/ha) and two sprays, the first at 45 days after germination and the second at 90 days after germination, recorded the disease incidence of 12%, which accounted for the disease reduction by 60%. This was on par with the basal application of *P. fluorescens* (soil application @ 2.5 kg/ha) which recorded the disease incidence of 13%. This was followed by the basal application of zinc sulphate @ 25 kg/ha + zinc sulphate 0.5% spray (twice) and the disease incidence of 13% was recorded. Plant products such as Mahua cake reduced both Macrophomina blight and tuber rot incidence by more than 50% compared to the oil formulation (Table 3). This study encourages the use of the biocontrol agent for a reduction in the Macrophomina blight and

Table 4. Colchicine content in *Gloriosa* seed

Sample No.	Treatments	Colchicine (%)
1	Mahua oil 10% (two sprays)	0.26
2	Basal soil application of *T. viride* @ 2.5 kg/ha and spray with *T. viride* 0.2%	0.32
3	Basal soil application of *P. fluorescens* @ 2.5 kg/ha and spray with *P. fluorescens* 0.2%	0.35
4	Basal application of *P. fluorescens* and *T. viride* and spray with 0.5 % zinc sulphate	0.37
5	Basal application of iluppai cake @150 kg/ha and spray with iluppai oil 3% (2 sprays)	0.07
6	Carbendazim 0.1% drenching (45 days) and one foliar spray (90 days)	0.24
7	Control	0.20

tuber rot incidence. The efficacy of antagonists in the control of *M. phaseolina* was reported earlier in pigeon pea (Lokesha & Benagi 2007), eggplant (Ramezani 2008) and sunflower (Dawar *et al*. 2008).

Efficacy of boicontrol agent and plant products on colchicine content (%)

A mixture of alkaloids consisting mainly of colchicine from dried tubers of *G. superba* was isolated. Colchicine levels in *G. superba* corms on the level of around 0.2% were reported earlier (Bharathi *et al.* 2002). In our study the colchicine level varied with the treatments (Table 4). However, a higher colchicine content was recorded in corms treated with biocontrol agents.

To conclude the application of biocontrol agents is attributed to the healthy growth of *G. superba* crops directly inhibiting the fungal growth and its activity and ultimately boosting the colchicine content, which results in good quality tubers with increased yield.

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Received for publication April 13, 2011
Accepted after corrections December 20, 2012

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