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RESEARCH ARTICLE

Strategies for the Management of Leaf Blight Disease Caused by *Alternaria alternata* in *Gloriosa superba* (L.)

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ABSTRACT

Leaf blight caused by *Alternaria alternata* (Fr.) Keissler is a severe disease of Gloriosa, causing severe yield loss in every part of Gloriosa growing areas of Tamil Nadu. The foliar pathogen was isolated from disease-infected leaves and proved its pathogenicity. Further, the molecular analysis of the pathogen using 18S rDNA confirmed the pathogen as *Alternaria alternata*. Attempts were made to explore the biocontrol agents and fungicides for the management of leaf blight incidence under field conditions. Four field trials conducted from 2014 to 2018 revealed that foliar application of talc-based formulation of *Bacillus subtilis* significantly reduced the leaf blight incidence and increased the seed yield under field conditions. Besides, prophylactic application of these biocontrol agents has also increased the plant growth parameters like plant height, number of flowers/plant, no. of pods/plant and number of seeds/pod. Similarly, foliar application of chlorothalonil (0.1%) and mancozeb (0.2%) was also credited to managing the leaf blight disease under field conditions.

Keywords: *Alternaria leaf blight; PCR analysis; Biocontrol agents; Fungicides; Disease suppression period.*

INTRODUCTION

Gloriosa superba (L.) an important medicinal crop belongs to Liliaceae family, is an herbaceous and climbing perennial plant commonly known as Climbing-lily, Glory-lily, and Gloriosa lily, and Flame-lily. It comprises rich source of active alkaloid, Colchicine in all the parts, especially the tubers that contain more alkaloid. Colchicine is used to treat the number of ailments such as gout, piles and various skin problems. The plant also contains another alkaloid, glorioside, in its flowers. The major growing states are Maharastra, Karnataka, Kerala, Assam, Tamil Nadu and Goa. Owing to its potential medicinal value, medicinal and aromatic plants are cultivated in an area of 10,038 hectares during 2018, especially *Gloriosa superba* is cultivated in an area of 3,336 hectares in Tamil Nadu (Department of Horticulture and Plantation Crops, 2018). The export potential of the seeds, which have high medicinal value is around 700 crores. The plant is also the state flower of Tamil Nadu and it is propagated from its underground rhizomes. The plant is affected by many pests and diseases, which are found to be a serious problem in the cultivation of the Gloriosa. The major diseases occurring in Gloriosa are leaf blight and tuber or basal stem rot and wilt. Among the diseases, the leaf blight caused by *Alternaria aternata* (Maiti *et al*.,2007) is found to be a serious disease and cause comprehensive yield loss (Thiribhuvanamala *et al*., 2020). The higher incidence of leaf blight disease occurs during cloudy weather coupled with high humidity. Owing to its continuous cultivation and suitable environmental conditions favoring pathogen that leads to disease development. The initial symptoms of leaf blight appeared as small, circular to oval, light brownish spots, visible with scattered manner at the tip, margin, and midrib of the leaves. Later, small spots are coalesced to form large spots and usually developed into a concentric ring. At the severe stage, the spots became dark brown to blackish in color, gradually coalesced and became irregular in shape, then the affected leaf blighted completely (Chandan Kumar *et al*., 2007).

Fungal diseases could be precisely managed using different approaches like cultural, physical, chemical, and biological methods. However, each method has its own merits, like chemical methods have immediate action on the pathogens, biological method has ecofriendly in nature and beneficial to soil microbes. Though these methods also have demerits when applied separately, including residual toxicity and environmental pollution and development fungicide resistance in pathogen in case of chemical

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method, the establishment of beneficial microbes in the infection court of pathogen has taken longer period. In order to mitigate the demerits of the individual method, a combined application in terms of integrated disease management (IDM) might be the best choice of management of leaf blight of Gloriosa. Since the application of fungicide alone is difficult for the management of leaf blight pathogen because of its broad host range and development of resistance in fungal pathogen, and residual problem in the seeds. To overcome the resistance in fungal pathogen, application of beneficial soil microorganisms, including plant growth promoting rhizobacteria (PGPR), could help manage the disease under field conditions. Among the PGPR, *Pseudomonas fluorescens* and *Bacillus subtilis* have been well explored for the management of many fungal diseases in crop plants. However, the native antagonists against leaf blight of Gloriosa has not been reported for the effective management of leaf blight. The antifungal properties of beneficial microbe *Bacillus subtilis* was attained by the presence of antimicrobial lipopeptide genes including iturin, surfactin, fengycin and bacillomycin D in *Bacillus*. Similarly, *Pseudomonas fluorescens* expressed the antimicrobial lipopeptide genes, including the 2,4-diacetylphloroglucinol (DAPG) and phloroglucinol. In addition, PGPR aiding in an extensive mechanism such as quorum sensing (QS) and signal interference, exhibiting antimicrobial activity, production of volatile organic compounds (VOCs), induction of systemic resistance, promotion of beneficial plant-microbe symbiosis and interference with pathogen toxin production (Bhattacharyya and Jha, 2012). These also reduced the disease progress both directly and indirectly through the production of antimicrobial lipopeptide, including iturin, surfactin, fengycin and bacillomycin D from *Bacillus* and DAPG and phloroglucinol from *Pseudomonas fluorescens*. These organisms induced the plant defense response termed induced systemic resistance through microbe-associated molecular pattern triggered immunity in crop plants (Yang *et al*., 2009). Previous findings indicated that *Bacillus* and *Pseudomonas* strains have a greater capacity to produce compounds such as lipopeptides and secondary metabolites, which manifest antimicrobial activities against plant pathogens (Cheng *et al*., 2016). Similarly, the plant extracts have been effectively utilized for the plant disease management merely with the wider mechanism of action including the induced the plant defense to suppress the growth of the pathogen, showed a direct effect on the pathogen and use of plant extract are cheap, locally available, non-toxic and biodegradable. Considering the importance of the disease, *B. subtilis, P. fluorescens*, plant extracts, and fungicides have been evaluated for the management of leaf blight of Gloriosa under field conditions.

MATERIAL AND METHODS

Isolation of pathogen from infected Gloriosa

The leaf blight pathogen was isolated from infected Gloriosa leaves on potato dextrose agar (PDA) medium by tissue segment method (Rangaswami, 1958). The infected leaves were cut into small pieces of 1.0 to 1.5 cm, surface sterilized with 0.1 per cent mercuric chloride for one min. and washed in sterile distilled water thrice and blot dried with sterilized filter paper. Then the leaf bits were placed in Petri plates containing potato dextrose agar (PDA) medium. The plates were incubated at 28 ± 2 °C for four days and observed for the fungal growth. The fungus was purified by a single spore isolation technique (Riker and Riker, 1936) and the purified isolate was maintained on PDA slants for further studies. The pathogen was identified up to species level based on their cultural and morphological characters and observed with an image analyzer under 100x magnifications for the presence of conidia and conidiophores. The conidia of

A. alternata were muriform in shape with dark brown color.

Pathogenicity test

The pathogenicity of purified fungal pathogen *A. alternata* was confirmed by Koch's Postulates. Two months old gloriosa seedlings were maintained in glass house in mud pots at 28±2 °C and at 80 per cent RH. The potting mixture consist, two parts of laterite soil, onepart of sand and one part of well decomposed farm yard manure. The experiment was maintained with three replications comprising of five plants per replication. The conidial suspension ($5x10^5$ spores mL^{-1}) was prepared in phosphate buffer (pH-7) from nine day old PDA cultures of the different isolates. The spore suspensions were sprayed on to the gloriosa seedlings. The inoculation was done during cool evening hours. The seedlings sprayed with sterile distilled water served as control and plants were kept up to 30 days for symptom expression. The symptoms were observed and compared with the initial symptoms. The fungus was re-isolated from artificially inoculated gloriosa leaves and compared with original pathogen.

Detection of genus-specific ITS region of A. alternata

To confirm the isolates as Alternaria 18S rDNA specific primers ITS-F (5'-GTCCTAACAAGGTTTCCGTA-3';) and ITS-R (5'-TTCTCCGCTTATTGATATGC-3') were used to get 650 bp PCR amplicon of ITS region (Dong *et al*., 2002). PCR was executed with a 50 µL reaction

volume of the following solution mixture: (1 ng of template DNA, 0.2 mM each dATP, dGTP, dCTP, and dTTP, 50 pcM of primer, 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 per cent Tween 20, 0.5per cent Nonidet P-40, 50 per cent glycerol, one unit of Taq DNA polymerase (Takara, JAPAN) and 5 mM MgCl). PCR amplifications were performed in a thermal cycler (Eppendorf Master Cycler Gradient, Westbury, New York, USA) and denaturation was executed at 94 °C for three min. before PCR cycling. The reaction cycle consisted of 40 sec at 94 °C for denaturation, 60 sec at 55 °C for annealing, and 60 sec at 72 °C for extension. A total of 35 cycles was performed. The final extension was done at 72 °C for 10 min. The PCR products were resolved on 1.2 % agarose at 50 V stained with ethidium bromide (0.5 µg mL^{-1}) and photographed using gel documentation system (Alpha Innotech Corporation, San Leandro, California). Amplified 18S rDNA was purified from each reaction mixture by agarose (1.2%, w/v) gel electrophoresis in TBE buffer containing 0.5 µg of ethidium bromide per mL, gel was excised and purified by using a QIA quick gel extraction kit (Qiagen, Inc., Chatsworth, California). The PCR product was subjected to sequencing and sequencing was performed at Bioserve Biotechnologies Pvt. Ltd, Genome Valley, Hyderabad, India. The rDNA homology searches were performed using the BLAST program (Altschul *et al*., 1990) through the internet server at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, USA).

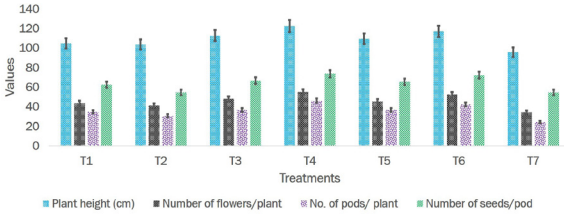

Figure 1. Plant growth promotion of Gloriosa by biocontrol agents, plant extract and fungicides under field cultivation. Error bars indicate standard deviation obtained from three replicates per treatment.

Management of leaf blight under field conditions

The field trials were conducted during the year 2014-18 at Sirumugai, Coimbatore district to test the efficacy of *P. fluorescens, B. subtilis*, lemongrass, and fungicides like mancozeb and chlorothalonil against leaf blight. Gloriosa tubers were planted in raised beds of 60 cm width and 8-meter length. Each tuber was placed at 30 cm depth on the raised bed. Four beds formed one plot. Averages of 100 plants were raised on each plot. The trials were laid out in a randomized block design (RBD) with three replications. The package of practices was followed as per the farmer practices and the treatments were given as described below on 30 and 60 days after planting (DAP) since the disease incidence begins from September to mid-November, which coincides with flowering.

Treatments	
T_1	Spraying *Pseudomonas fluorescens* 0.2% on 30 DAP & 60 DAP
T_2	Spraying EC formulated lemongrass oil 0.2% on 30 DAP & 60 DAP
T_3	Spraying *Pseudomonas fluorescens* 0.2% on 30 DAP and lemongrass oil 0.2% on 60 DAP
T_4	Spraying *Bacillus subtilis* (0.2%) on 30 DAP & 60 DAP
T_5	Spraying mancozeb 0.2% on 30 DAP & 60 DAP
T_6	Spraying chlorothalonil 0.1% on 30 DAP & 60 DAP
T_7	Control

The observation on the disease incidence was recorded before the initiation of spray, one week after first spray and second spray. The severity of anthracnose disease was recorded on 10 plants selected at random in each replication of the treatment. Percent Disease Index (PDI) was calculated using standard score chart (0-9 scale) (Pawelec *et al*., 2006) and the yield was also recorded.

Statistical analysis

Mean differences of the treatment were evaluated with ANOVA using Duncan's Multiple Range-Test at 5% significance (Gomez and Gomez, 1984). All the data were statistically analyzed with IRRISTAT (version. 3/93, Biometrics unit, International Rice Research Institute) and consequently interpreted.

RESULTS AND DISCUSSION

Owning to the pharmaceutical importance, Gloriosa is being cultivated in Tamil Nadu and other parts of India to meet out the needs of current scenario. The extensive cultivation of Gloriosa has resulted in the severe outbreak of leaf blight disease, which causes considerable yield loss. In the present study, infected leaves showing typical symptoms of leaf blight were collected from farmers' field at Sirumugai, Coimbatore district of Tamil Nadu to find out the pathogenic fungi involved in leaf blight. The pathogen was isolated on the PDA medium using the infected leaves and the morphological characters of the isolated fungus were pertinent to *A. alternata* as per the original descriptions given by Nees (1817) and quoted by Wallace (1929) and Wiltshire (1933). The pathogenicity was proved on 3 months old Gloriosa plant maintained under glass house conditions by following the pin prick method. The results revealed that inoculated plant produced characteristic symptoms of leaf blight, including water-soaked lesions and circular necrotic

spots, after 15 days of inoculation. However, mock-inoculated plants have not shown any symptoms of leaf blight under glasshouse. The Koch's postulates were fulfilled by consistently re-isolating *A. alternata* from inoculated leaves of Gloriosa plants. Further, to confirm the association of *A. alternata* as a causative agent of leaf blight of Gloriosa, PCR reaction was performed using ITS region of Alternaria corresponding to 18S rDNA region. The DNA isolated from Alternaria was amplified to a fragment size of 550 bp, products were resolved on a 1.2% agarose gel and their sequence was determined. NCBI-BLAST analysis of the nucleotide sequences showed 100% nucleotide homology with those of other *A. alternata* present in the GenBank Database. The results of BLAST searches revealed the presence of

Table 1. Efficacy of bacterial antagonist, plant extract and fungicides against leaf blight of Gloriosa under field cultivation.

Treatments	2014-15		2015-16		2016-17		2017-18		Pooled Mean	
	PDI	% disease reduction over control	PDI	% disease reduction over control	PDI	% disease reduction over control	PDI	% disease reduction over control	PDI*	% disease reduction over control
T_1	22.1	19.3	22.9	31.4	21.4	27.7	15.5	46.4	20.5	31.2
	(27.8)		(23.1)		(22.3)		(23.2)		(24.1)	
T_2	23.4	14.6	23.2	30.5	24.7	16.6	17.0	41.3	22.1	25.8
	(28.6)		(23.6)		(23.8)		(24.3)		(25.1)	
T_3	19.4	29.2	19.8	40.7	20.1	32.1	18.5	36.2	19.5	34.6
	(26.2)		(21.2)		(21.6)		(25.4)		(23.6)	
T_4	18.2	33.6	17.9	46.4	16.2	45.3	10.8	62.8	15.8	47.0
	(25.4)		(20.1)		(18.4)		(19.2)		(20.8)	
T_5	20.3	25.9	21.9	34.4	22.5	23.9	10.3	64.5	18.8	37.1
	(26.7)		(22.6)		(22.9)		(18.7)		(22.7)	
T_6	17.3	36.8	18.7	44.1	17.4	41.2	11.4	60.8	16.2	45.7
	(24.1)		(20.5)		(19.2)		(19.7)		(20.8)	
T_7	27.4	-	33.4	-	29.6	-	23.3	-	28.4	-
	(29.4)		(29.1)		(27.1)		(28.8)		(28.6)	
SEd	0.81		0.85		1.05		0.7		0.85	
CD (P=.05)	1.6		1.8		2.1		1.53		1.75	

*- Pooled mean data of four seasons

A. alternata as causative agent of leaf blight in gloriosa. The sequence of this isolate was submitted in NCBI database, bearing the accession number MN894079. Since ITS region (18S rDNA region) of the nuclear ribosomal repeat unit is used for molecular identification and primary genetic marker for species-level detections in several fungal pathogens. Dong *et al*. (2002) reported the amplification of ITS region of *A. brassicola* with ITS-F and ITS-R specific primer yielded 650 bp amplicon and which confirmed genus the Alternaria. Prathima *et al*. (2018) amplified the leaf blight pathogen A. alternata from marigold through PCR using 18S rDNA specific primers and which had 99% to 100% similarity with existing isolates. Jankar *et al*. (2018) also amplified the ITS region of *A. alternata*, causing fruit rot of chilli with an amplicon size of 440 bp using the PCR technique. Similarly, the 18S ribosomal RNA gene of *A. alternata* causing brown rot disease of lemon showed 100% nucleotide sequence similarity with *A. alternata* NS8 (Liaquat *et al*., 2021).

The management of plant diseases using different formulations of *Bacillus spp., Pseudomonas spp*. have been reported previously (Kloepper and Schroth, 1981; Radjacommare *et al*., 2002; Rajamanickam *et al*., 2018). The biological control of plant disease is more preferable nowadays since it is an alternative method to chemical management, safe to the environment and ecofriendly. In the present study, field trials were conducted to test the efficacy of biocontrol agents, plant extract and fungicides against leaf blight of Gloriosa. The results revealed that spray application of talc-based formulation of *B. subtilis* (0.2%) at 30 DAP and 60 DAP was found to be effective in reducing the leaf

blight incidence, which accounted to 15.8 PDI and 47.0 per cent disease reduction over control against the untreated control plot accounted 28.4 PDI (Table 1). In addition, the highest dry seed yield of 514 kg/ha accounting for 27 per cent increase over control in the *B. subtilis* treated plants and increased the plant height (122.6 cm), number of flowers/plant (55 Nos.), number of pods/plant (46 Nos.) and number of seeds/pod (73 Nos.) were also observed (Figure 2). In general, plants applied with *B. subtilis* influenced plant growth promotion, which showed maximum plant growth promotion in Gloriosa. Similarly type of results was observed by Priyanka *et al*. (2018) who found that foliar application *B. subtilis* subsp. *spizizenii* (MM19) effectively suppressed the leaf blight of marigold caused by *A. alternata* under field conditions up to 77 per cent. Further, they revealed that the antifungal activity of *B. subtilis* subsp. *spizizenii* (MM19) due to the presence of Phthalic acid esters, compound involved in the inhibition of fungal pathogen. Rajamanickam *et al*. (2018) found that soil application of *B. subtilis* PP and CL3 strains showed the plant growth promotion activities in banana and increased activity of defense enzymes like peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), phenolic content and PR proteins; chitinase and β-1,3-glucanase were also observed as an induced defense mechanism against the pathogen. Similarly, the bacterial antagonist *Pseudomonas* and *Bacillus* spp. showed the plant growth promotion activities in tomato (Ramakrishna *et al*., 2018). Basamma and Shripad Kulkarni (2017) found that foliar application of *B. subtilis* at 10g/L reduced the early blight incidence in tomato caused *A. alternata* and increased the plant growth promotion by recording the highest number of branches, fruits and fruit weight per plant. Ahmed (2017) tested the efficacy of two bacterial antagonists, namely *Brevibacillus formosus* strain DSM 9885 and *Brevibacillus brevis* strain NBRC 15304, against the brown leaf spot of potato under greenhouse condition. The combined application of these two antagonists was found to be superior in their efficacy against disease than the individual applications. They also found the presence of new protein profiles from the plants applied with two antagonists resulted in induce defense mechanisms.

In addition, combined application of *P. fluorescens* and lemongrass oil recorded the leaf blight incidence of 19.5 PDI, which accounted 34.6 per cent disease reduction over control. The least inhibition was noticed in spray application of EC formulated lemongrass oil (0.2%) at 30 DAP and 60 DAP, which accounted disease incidence of 22.1 PDI and 25.8 per cent disease reduction over control, respectively. This is in agreement with Moges *et al*. (2012), who found that the application of *P. fluorescens* TK3 effectively reduced the disease incidence of early blight of tomato under greenhouse conditions and increased the plant growth promotion, resulted in increased plant height and biomass of tomato. Similarly, Sitara *et al*. (2008) reported that the Nigella sativa oil at 0.15% and 0.1% completely inhibited *Aspergilus niger, Drechslera hawiensis, Alternaria alternata* and *Fusarium moniliforme* and Neem oil showed greater suppression of the growth of *Drechslera hawiinesis* and *Alternaria alternata* at 0.15% and 0.1% concentration. The plants applied with extracts of Datura metal showed increased activity of defense-related enzymes like peroxidase, polyphenol oxidase in pathogen-infected chilli plants (Rajamanickam *et al*., 2016). Several methods, including host resistance, chemical and biological approaches, have been practiced for several decades for the management of plant diseases. However, among several methods developed for the management of disease, chemical control has been widely practiced in several crops. In the present study, spray application of chlorothalonil (0.1%) at 30 DAP and 60 DAP and spray application of mancozeb (0.2%) at 30 DAP and 60 DAP recorded the leaf blight incidence of 16.2 PDI and 18.8 PDI, respectively. Similarly, Mahadev *et al*. (2018) found that foliar application of Hexaconazole (0.1%) reduced the leaf spot and flower blight incidence in marigold caused by *A. tagetica*. The lowest disease incidence of 32.15 PDI on leaves and 33.76 PDI on the flower was observed in plants applied with Hexaconazole (0.1%) followed by mancozeb (0.2%). Similarly, higher follower yield was observed in plants applied with Hexaconazole (0.1%) and Mancozeb (0.2%) to the maximum of 6.96 t/ha and 6.81 t/ha, respectively. Rasheed *et al*. (2019) screened the four fungicides like carbendazim, copper oxychloride, Aerosal and score against *A. alternata* causing leaf spot disease of Aloe vera under in vitro and in green house conditions. They found that maximum disease reduction was observed with the application of score to the maximum of 17.7 per cent under greenhouse conditions.

CONCLUSION

Foliar application of talc-based formulation of *B. subtilis* could be the effective management strategy for the management of leaf blight of Gloriosa caused by *A. alternata* under field conditions. The application of chlorothalonil (0.1%) and mancozeb (0.2%) was also found effective in reducing the leaf blight incidence. However, the application of fungicides may cause residual problems; hence prophylactic application of *B. subtilis* not only plays a major role in inducing disease resistance but also promotes plant growth and leads to yield enhancement.

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