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Biological Management of Leaf Blight Disease of an Endangered Medicinal Plant.

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ABSTRACT

Gloriosa superba Linn. (Glory lily) is an endangered medicinal plant belonging to the family Liliaceae. A leaf blight disease caused by the fungus *Alternaria alternata* was observed in different areas of lower Gangetic plains of West Bengal, India. *Pseudomonas aeruginosa* strain WS-1 showed both *in vitro* and *in vivo* antagonistic activity against the pathogen. In dual culture bioassay as circular and semicircular patterns, the isolate quantitatively inhibits the growth of the pathogen by about 77% and 67%, respectively. In talc-based formulation, the strain has adequate shelf life. Experimental data illustrates about 70% survivability of the population after 180 days of storage at 4°C. Foliar application of a talc based formulation of *P. aeruginosa* strain WS-1 to field condition revealed that the maximum mean disease index reached 1.17 and 1.06 in 2012 and 2013 respectively, indicating 75% and 78% reduction in disease severity respectively when compared to control. Application of the outcome of this work in the commercial cultivation would benefit the growers by facilitating bioorganic production of this important medicinal plant and finally the consumers will tend to receive a pesticide free natural product.

Keywords: *Alternaria alternata,* biocontrol, *Gloriosa superba*, *Pseudomonas aeroginosa* WS-1.

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INTRODUCTION

Gloriosa superba Linn. also known as glory lily belongs to the family Liliaceae. It is one of the endangered species among the medicinal plants [1]. Different types of phytochemicals are found in glory lily [2]. However, in the world market they are considered as rich source of colchicine and gloriosine [3]. These two phytochemicals are commonly used for treatment of gout and rheumatism. Phytochemicals from glory lily have wide antimicrobial spectrum against Gram-positive and Gram-negative bacteria along with antifungal and mutagenic potential [4]. The presence of mannose derivative and oligomannose carbohydrate has shown antiviral potential [5]. Various chemical fractions of glory lily have potent neutralizing effect of rattlesnake venom, when administered subcutaneously to mice [6]. It is also used to treat intestinal worms, skin diseases and various respiratory disorders [7].

Phytopathogens attack medicinal and aromatic plants leading to significant quantitative and qualitative loss but most of the diseases are of fungal origin [8]. *Alternaria* blights were very common in medicinal plants cultivated in various districts of West Bengal, India [9,10,11,12]. Today biocontrol agents are gaining importance in the field of disease management of medicinal plants as they do not have the adverse effects like that of fungicides which eventually contaminate the purity of plant drugs [13]. Extensive research is underway globally to exploit the potentiality of pseudomonads, which help to protect crops from phytopathogens and are metabolically and functionally more diverse [14]. A wide range of fluorescent pseudomonads have been reported for having *in vitro* and *in vivo* biocontrol potentiality against variety of phytopathogens [15,16,17,18,19]. Here, attempts have been made to evaluate antagonistic activity of *Pseudomonas aeruginosa* strain WS-1 against leaf blight disease of *Gloriosa superba.*

MATERIALS AND METHODS

Organisms

The pathogenic organism was isolated from the diseased leaves of *G. superba* as a pure culture on potato dextrose agar medium (PDA), identified as *Alternaria alternata* [11]. The culture was maintained in the same medium and stored at 4°C for further study. The biocontrol agent *P. aeruginosa* strain WS-1 (MTCC # 8158) was obtained from our laboratory culture stock. The antagonist was subcultured and maintained on tryptic soy agar (TSA) medium for subsequent use.

In vitro antagonistic bioassay

The antagonist from 24 h old culture (10^7 cells /ml) was streaked in the peptone glucose agar (PGA) plate as circular / O and semicircular / U pattern. Then mycelial disc (5 mm diameter) of 3 days old culture of *A. alternata* was subsequently inoculated at the center of O or U shaped region on the PGA plates [20]. Inoculation only with the pathogen served as control. The plates in triplicate were incubated at 30°C for 5 days and diameter of colony

growth was measured at every 24 h intervals. Light microscopic (Zeiss AX 10) studies were also performed to detect physical and / or morphological changes of mycelia.

Survivability of *P. aeruginosa* WS-1

In talc based formulation

Talc based formulation of the antagonist was prepared using the method developed by Vidyasekaran and Muthamilan (1995) [21]. *P. aeruginosa* WS-1 was found to be tolerant against streptomycin at 50 µg/ ml. The isolate was grown in Kings B medium supplemented with streptomycin (50 µg/ ml) for 48 h on a rotary shaker (150 rpm) at 30°C. The bacterial suspension (8×10^9 colony forming unit (CFU/ ml) was mixed with sterile talc (400ml/ kg) containing CMC (10g/ kg) and air dried (approximately to 35% w/w, moisture content). Survival of the bacterial population in the formulation was assayed at 30-day intervals for 180 days using King's medium B by dilution plating.

On phylloplane

Survival and multiplication of the antagonist on the phylloplane of *G. superba* was determined following the method of Kishore *et al.* (2005) [15]. Colonies of WS-1 was determined after 48h of incubation at 30°C utilizing its colony morphology, fluorescence and streptomycin tolerant characteristics. The bacterial populations were expressed as log CFU/ g of leaf.

Field studies

The Medicinal Plant Garden, R.K. Mission Ashrama, Narendrapur, India was used for the field experiments during 2011 and 2012 when the environmental conditions were conducive (February to July) for the rapid spread of *A. alternata* on *G. superba.* The trial was conducted as a randomized complete block design with three replicate plots (3×4 m^2) and forty plants per plot. Well-rotted farmyard manure was mixed well into the soil before planting the saplings. Thirty-day-old disease free saplings were transplanted to the random blocks on mid February allowing *Alternaria* leaf blight to develop naturally [22]. The talc based formulation of *P. aeruginosa* WS-1 was prepared by dissolving it in water (4 g/ l) allowing it to settle for 1 h, and filtering the solution through muslin cloth. The filtrate was applied as a foliar spray using a low volume sprayer beginning at transplant and repeating every 15 days until harvesting i.e. up to the end of July 2012 and 2013. Plots sprayed with the talc-based carrier without the biocontrol agent was served as control. Thirty plants from each plot were rated for disease severity at 15 day intervals starting at transplant until harvest using a 0-5 rating scale [15].

Statistical analysis

The disease severity data were statistical analysed by using analysis of variance (ANOVA) followed by Tukey's Test to find out significance level at 1% ($p<0.01$).

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RESULTS

Interaction of *P. aeruginosa* WS-1 against *A. alternata* in dual culture

Significant growth inhibition of *A. alternata* by the strain WS-1 was observed in dual culture. The growth inhibition of *A. alternata* remained proportionate with an increased incubation period of up to 5 days. Quantitatively the antagonist inhibited the growth of the pathogen by 76.75% and 67.34% in circular and semicircular streaks after 120 h of incubation respectively (Figure 1). Microscopic examination of the mycelia at the interaction zone showed signs of shriveling, growth deformities, swelling, fragmentation, short branching and lysis (Figure 2).

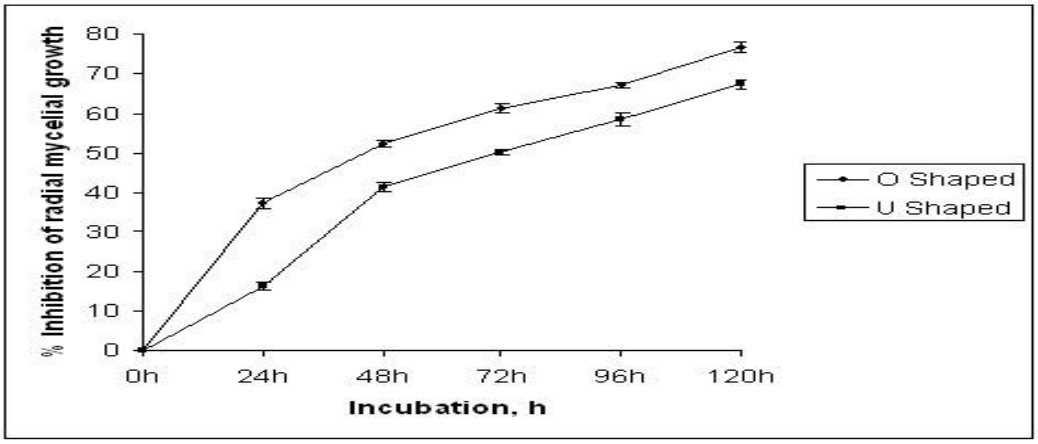

Figure 1: Inhibition of *A. alternata* by *P. aeruginosa* WS-1 under dual plate culture using circular (O) and semicircular (U) method. Each point represents the mean ± SE (standard error) of three separate experiments, each in triplicate

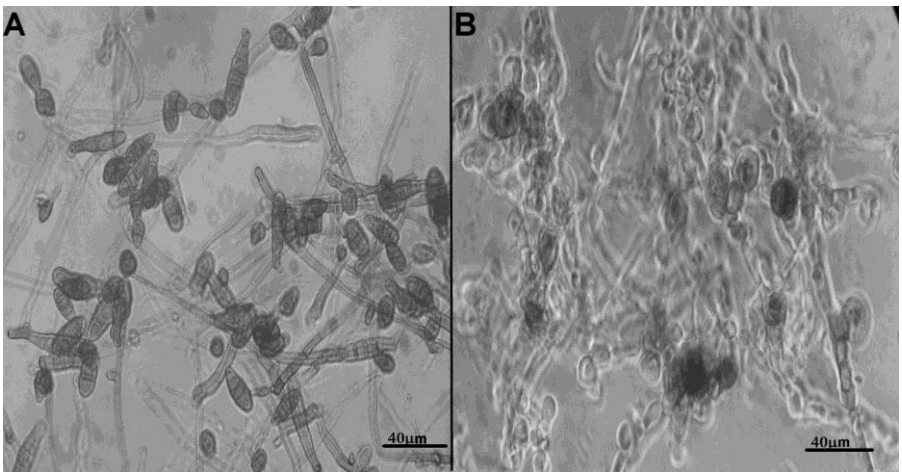

Figure 2: [A] Microscopic observation of mycelium of control plate showing normal hyphal structure and spore [B] Microscopic observation of mycelium of treated plate showing hyphal shriveling, short branching, hyphal deformities and hyphal tip swelling

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Survival of *P. aeruginosa* WS-1 in talc-based formulation and on the *G. superba* phylloplane

In talc based formulation, the survival of WS-1 was monitored for 180 days (Figure 3). With time, the initial population of WS-1 (8.3 log CFU/ g) in talc based formulation gradually decreased. A total of approximately 30% decrease in the colony forming unit was estimated on 180th day after storage at 4°C. Foliar application of this formulated antagonist showed the initial population (within 1 h after application) to be log 6.4 CFU/ g of leaves. The initial population steadily declined to log 3.5 CFU/ g of leaves at 15th day after treatment (Figure 4). No colonies of WS-1 were recovered on the controls.

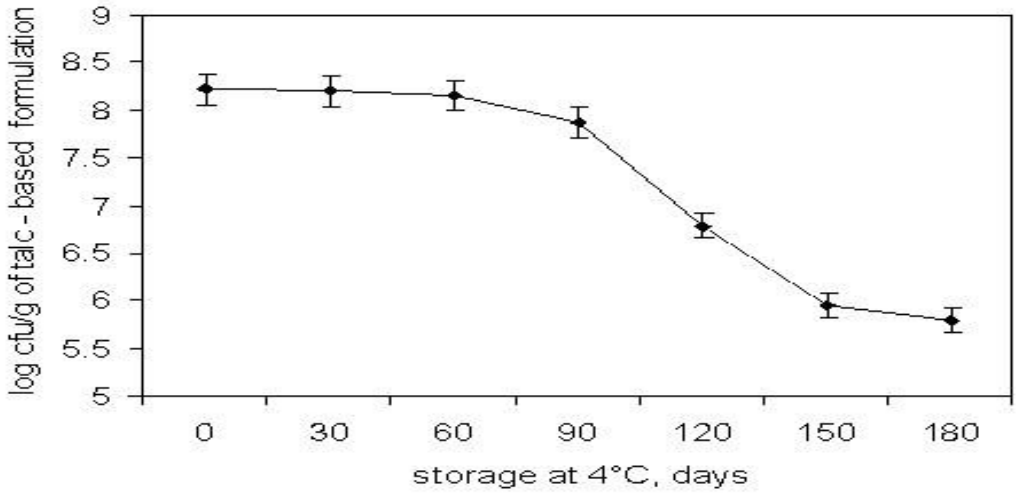

Figure 3 Survival of *P. aeruginosa* WS-1 in talc based formulations stored at 4°C. Each point represents the mean ± SE (standard error) of three separate experiments, each in triplicate

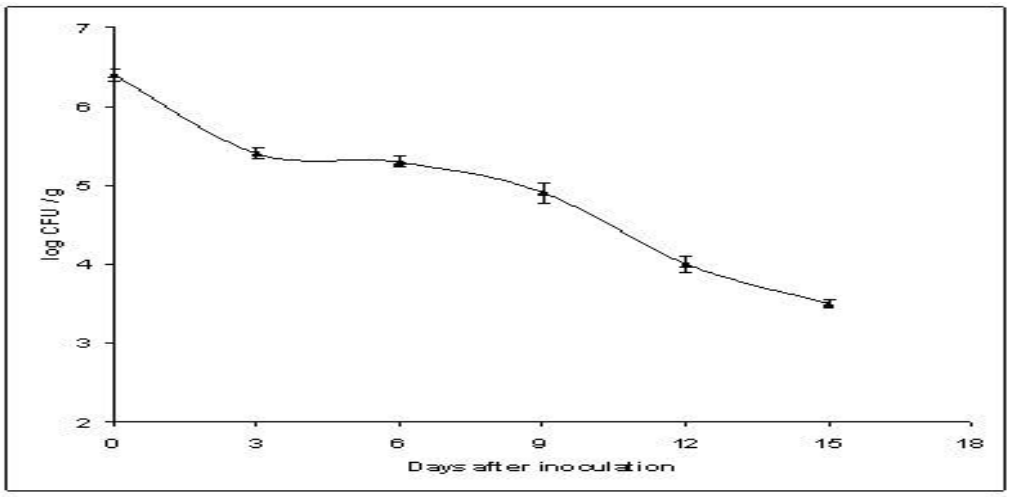

Figure 4 Changes in the populations of *P. aeruginosa* WS-1 on the phylloplane of *G. superba* growing under field condition. Each point represents the mean ± SE (standard error) of three separate experiments, each in triplicate

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Field studies

Talc based formulation of *P. aeruginosa* WS-1 was evaluated in the field on *G. superba* for two successive seasons. After the 2nd and 3rd spray of WS-1, new symptoms of *Alternaria* leaf blights were inhibited in treated plots. At the time of harvest, the mean disease index in control field reached to 4.68 and 4.74 in 2012 and 2013 respectively, where more or less all plants were severely affected and more than 50% leaves were defoliated (Table 1). On the contrary, the disease index in WS-1 treated fields at harvest reached only 1.17 and 1.06 in 2012 and 2013 respectively, indicating 75% and 78% reduction in disease severity respectively.

Table 1 Efficacy of talc based formulation of *P. aeruginosa* WS-1 for the control of leaf blight disease of *G. superba* caused by *A. alternata* in 2011 and 2012. Talc based formulation was applied as foliar sprays on date of transplantation and at an interval of 15 days until 180 days. Disease index was rated on a 0 - 5 scale [15]. Values are mean ± SE of thirty randomly selected plants per plot of three individual plot experiments

Days after transplantation	Disease index 2011		Disease index 2012	
	Control	Treated	Control	Treated
0	0	0	0	0
15	0.364±0.009	0.252±0.005*	0.303±0.008	0.262±0.009*
30	0.738±0.008	0.364±0.007*	0.714±0.024	0.388±0.006*
45	1.12±0.006	0.505±0.014*	1.36±0.026	0.521±0.021*
60	1.548±0.011	0.754±0.012*	1.577±0.014	0.652±0.009*
75	2.225±0.014	0.83±0.024*	2.11±0.051	0.675±0.016*
90	2.95±0.012	0.865±0.022*	2.83±0.019	0.691±0.024*
105	3.4±0.015	0.91±0.012*	3.34±0.031	0.7±0.017*
120	3.9±0.011	0.954±0.015*	3.72±0.014	0.768±0.015*
135	4.02±0.021	0.99±0.018*	4.16±0.032	0.801±0.044*
150	4.2±0.041	1.12±0.016*	4.08±0.044	0.924±0.033*
165	4.32±0.033	1.15±0.012*	4.49±0.009	1.03±0.014*
180	4.68±0.019	1.17±0.012*	4.74±0.017	1.06±0.006*

* Data with an asterisk in each row differ significantly with control according to Tukey's test (P<0.001)

DISCUSSION

Over the past twenty years, the control of plant pathogenic fungi by antagonistic bacteria and fungi has been the topic of numerous studies. The majority of these studies dealt with antagonists controlling soil borne pathogens and to a less significant extent, foliar pathogens. *Pseudsomonas, Bacillus* and *Stenotrophomonas* have their ability to control phytopahogens both *in vitro* and on phyloplane [23,24]. Rozsnyay *et al.,* (1992) [25] showed that some strains of *P. fluorescens* and some fungi inhibited canker and dieback diseases of apricot. The first phylloplane biocontrol agent, *Pseudomonas fluorescens* A506, used to control fire blight and frost injury in apple and pears as well as it prevent blossom colonization by *Erwinia amylovora* [26,27].

Recent investigation on this antagonist demonstrated that *P. aeroginosa* WS-1 is a potent secretor of siderophore, volatile substances (hydrocyanic acid), proteases and chitinase

[28]. Furthermore, in a similar study *P. aeroginosa* WS-1 have the capacity to induce systemic resistance in *Capsicum annuum* when challenge inoculated with its pathogen *Colletotricum capsici* [29]. it was further established that the molecular mechanism behind the development of ISR was recognized as a potent signal molecule in case both systemic induced resistance [30] and ISR [31]. All this effect might have a synergistic role; there by the talc base formulated *P. aeroginosa* WS-1 performed so brilliantly at field level and control the leaf blight disease of *Glorosa superba* successfully up to 78%. The outcome of this experiment might help the farmers to limit the use of hazardous fungicides, simultaneously saving their crops and ultimately this benefit reach to the consumer through the medical practitioner and pharmaceutical industry.

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