

Management of leaf spot disease of *Stevia rebaudiana* Bertoni with antagonistic bacteria

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Abstract

Stevia rebaudiana Bertoni, a natural sweetener contains two main sweetest compounds, stevioside (ST) and rebaudioside A (R-A), tasting about 300 and 450 times sweeter than sucrose, respectively. This commercially important plant also suffers a leaf spot disease caused by the fungus *Alternaria alternata* in various districts of South Bengal, India. Fluorescent *Pseudomonas* BRL-1 isolated from the rhizosphere, 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 78% and 69%, respectively. *In vitro* plate assay detected that the strain produces hydrolytic enzymes, IAA and siderophore. 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 the talc based formulation of the antagonist in the field condition revealed that the maximum mean disease index reached to 0.653 and 0.663 in 2009 and 2010, which showed around 86% and 87 % reduction in disease severity, respectively when compared to control. Transferring the outcome of this work in the field would benefit the growers by facilitating bioorganic production of this important medicinal plant. Ultimately, the consumers will tend to receive a pesticide free natural sweetener.

Key words: *Alternaria alternata*, biocontrol, fluorescent *Pseudomonas* BRL-1.

Abbreviations: ANOVA, Analysis of variance; CAS, Chrome azurol S; CMC, Carboxymethyl cellulose; DMRT, Duncan's multiple range test; HCN, Hydrogen cyanide; IAA, Indole acetic acid; PDA, Potato dextrose agar; PGA, Peptone glucose agar; R-A, Rebaudioside A; ST, Stevioside; TSA, Tryptic soy agar.

Introduction

Stevia, botanically known as *Stevia rebaudiana* Bertoni (Asteraceae) is a sweet perennial herb, indigenous to the north-eastern corner of Paraguay (Kinghorn et al., 1984). *Stevia* is composed of several natural, heat-stable *ent*-kaurene glycosides (steviol glycosides) whose intensities of sweetness and flavour profiles differ from each other and vary according to concentration and environment (Geuns, 2003). The two main alkaloids being stevioside (ST) and rebaudioside A (R-A) are the sweetest compounds and tasting about 300 and 450 times sweeter than sucrose, respectively (Tanaka, 1982; Geuns, 2003). Stevioside have been used as a dietary supplement by the United States since 1995 (Brandle et al., 1998; Mizushina et al., 2005). Beside ST, *Stevia* also contains significant quantities of chlorogenic acid, which has hypoglycemic effects (Khramov and Dmitrienko, 2000; Gregersen et al., 2004). In addition, it has already been encouragingly established that the stevioside and benzopyran content in micropropagated *S. rebaudiana* amount to nearly the same as in the mother plants (Hwang, 2006; Supaibulwattana et al., 2011). Some ingredients of *Stevia* are commercially used as a low-caloric sweetener, i.e., as a sugar substitute. In India this commercially important medicinal plant has been introduced in the states of Maharashtra, Tamil Nadu, Karnataka, Rajasthan and West Bengal. Like other

cultivated plants, medicinal and aromatic plants are also attacked by a number of fungi, bacteria, viruses and nematodes leading to significant quantitative and qualitative loss. Most of the diseases are of fungal origin (Paul and Singh, 2002). Survey for the last five consecutive years confers that *Alternaria* leaf disease was very common in medicinal plants cultivated in various districts of West Bengal, India (Maiti et al., 2007a, b, c, d, e). The disease protection measures of medicinal plants are still restricted to the application of various chemical fungicides which strictly do not fit with the basic theory of usefulness of herbal drugs. Also, the residual effects of different chemicals eventually contaminate the purity of such plant drugs and are also of serious concern from environmental point of view (Sharma et al., 2004). Therefore, biological control agents are gaining importance in the field of disease management of medicinal plants (Mathivanan et al., 2005). The diversity of naturally occurring microorganisms of the rhizosphere and phyllosphere and their potential for biological control of plant pathogens have been examined extensively (Jayraj et al., 2007). Pseudomonads are considered to be important rhizosphere organisms, wherein considerable research is underway globally to exploit the potential. Fluorescent pseudomonads help in maintenance of soil health, protect crop from pathogens and are

metabolically and functionally more diverse (Choudhury et al., 2009). A number of fluorescent pseudomonads have been reported for having *in vitro* and *in vivo* biocontrol potentiality against wide range of phytopathogens (Fanny and Pfender 1997; Gupta et al., 2002; Kishore et al., 2005; Mansoor et al., 2007; Jayraj et al., 2007; Sen et al., 2009; Maiti et al., *in press*). Here, attempts have been made to evaluate *in vitro* and *in vivo* antagonistic activity of fluorescent *Pseudomonas* BRL-1 against the phytopathogenic fungi *A. alternata*.

Results

Interaction of fluorescent Pseudomonas BRL-1 against A. alternata in dual culture

In dual culture, significant growth inhibition of *A. alternata* by fluorescent *Pseudomonas* BRL-1 was observed. Mycelial growth was restricted near bacterial growth and continued away from it. The growth inhibition of *A. alternata* remained proportionate with an increased incubation period of upto 5 days. Quantitatively BRL-1 inhibited the growth of *A. alternata* 77.62 and 68.78% in circular and semicircular streaks after 120 h of incubation respectively (Fig 1). Microscopic examination of the mycelia at the interaction zone with fluorescent *Pseudomonas* BRL-1 showed signs of shriveling, growth deformities, swelling, fragmentation, short branching and lysis (Fig 2). *In vitro* assay for antifungal compounds: The observed morphological abnormalities of hyphae in dual culture suggested that fluorescent *Pseudomonas* BRL-1 produces secondary metabolites without fungal activity. Plate assay detected BRL-1 produces chitinase, protease, IAA, siderophore. The strain formed clear zones around colonies, when grown on chitin and gelatin amended media. The level of IAA was quantified spectrophotometrically. It was found that the isolate produce 24 µg / ml IAA at 48 h of incubation, increase in incubation period was proportionate to gradual decline in the production level of IAA upto 6 days. On CAS medium bacterial growth changed medium from blue to orange with the release of iron from the iron-dye complex indicating the production of a siderophore by BRL-1.

Survivability of fluorescent Pseudomonas BRL-1

a) In talc-based formulation

Survival of the antagonist in talc based formulation stored at 4°C was monitored for 180 days (Fig. 3). It was revealed that the initial population of BRL-1 (8.7 log cfu / g) in talc based formulation decreased gradually with time of incubation. However, there was no significant decrease in the viable population till 60 days of storage at 4°C. Following this, there was a sharp decline of the bacterial population resulting a survival of about 70% (5.81 log cfu / g) of the population after 180 days of storage at 4°C.

b) On phylloplane:

The talc-based formulation (4 g / l) of the antagonist was applied as foliar sprays with an initial cell density of 10⁶ cfu / g (log 6.6 cfu / g) of fresh leaves. The initial population was high after application but declined sharply with time. After 15 days

the population it was dropped down to log 3.6 cfu / g (Fig. 4). No bacterial colonies of BRL-1 were recovered on the controls.

Field studies

The talc based formulation of fluorescent *Pseudomonas* BRL-1 was evaluated at field condition successively for two seasons. Disease symptoms on leaf were assayed for six months after transplantation. Disease index was recorded every 15 days interval upto the date of harvest (Table 1). Data showed that after 2nd and 3rd spray the appearance of new symptoms more or less stopped in treated field. During the time of harvest the mean disease index in control field reached to 4.79 and 4.933 in the experimental year 2009 and 2010 respectively where more or less all plants were severely affected and more than 50% leaves were defoliated. In contrast, in treated fields during the time of harvesting the maximum mean disease index reached to 0.653 and 0.663 in the year 2009 and 2010, which showed around 86 and 87 % reduction in disease severity respectively.

Discussion

Disease management of medicinal plants needs to be focused on utilization of potential biocontrol agents. Control of plant pathogenic fungi by antagonistic bacteria and fungi have been extremely studied in the past two decades. Most of these studies dealt with antagonists controlling soil borne pathogens and to a lesser extent, foliar pathogens. An effort to characterize the mycoparasitic action of BRL-1 in the present study demonstrated that the bioagent form significant inhibition zone in dual culture. Observations of hyphal interaction between BRL-1 and pathogen revealed that the antagonist acted mainly by the release of secondary metabolites likes siderophore, IAA and lytic enzymes. These observations coincided with the findings by Lorito et al. (1994), Dunne et al. (1997) and Bano and Musarrat, (2002) who reported earlier that the exposure of selected phytopathogenic fungi to lytic enzymes such as chitinase, protease could result in the degradation of the structural matrix of the fungal cell wall. In general indole acetic acid is universally accepted as a plant growth promoter. Phytohormone could also induce resistance through PR protein production (Chirst and Mosinger 1989; Asselbergh et al. 2008); Sharaf and Farrag, (2004) reported that IAA reduce spore germination, mycelial dry weight and protein content of the pathogenic fungi and thus prevent significantly any chance for disease induction by soil pathogens. Our study complements their findings by demonstrating significant production of IAA by fluorescent *Pseudomonas* BRL-1. Siderophore production has been reported to protect tomato plants from *Pythium* by *P. aeruginosa* 7NSK2. According to several studies, siderophore, IAA, chitinase and protease produced by fluorescent pseudomonads are known to inhibit the growth of some fungal pathogens (Lim and Kim 1995; Bhatia et al., 2003). Fluorescent pseudomonads have been reported to survive in certain dry formulations (Connick, 1988, Vidhyasekaran and Muthamilan 1995; Sarma et al. 2011). It has been reported that the populations of fluorescent pseudomonads did not decrease in talc mixture with 20% xanthane gum after storage for four months at 4°C (Klopper and Schroth, 1981; Bora et al. 2004). In the present study, we have shown that BRL-1 could survive well in talc based formulation stored at 4°C for a period of 180 days. The success of any formulation of biocontrol agents not

Table 1. Efficacy of talc-based formulation of fluorescent *Pseudomonas* BRL-1 for the control of leaf spot disease of *S. rebaudiana* caused by *A. alternata* in 2009 and 2010. 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 (Kishore et al. 2005). Values are mean \pm SD of thirty randomly selected plants per plot of three individual plot experiments. Data with an asterisk in each row differ significantly with control according to Duncan's Multiple Range test (DMRT) ($P < 0.001$).

Days after transplantation	Disease index 2009		Disease index 2010	
	Control	Treated	Control	Treated
0	0	0	0	0
15	0.197 \pm 0.008	0.143 \pm 0.007	0.181 \pm 0.009	0.133 \pm 0.005
30	0.857 \pm 0.009	0.307 \pm 0.009*	0.744 \pm 0.022	0.279 \pm 0.006*
45	1.187 \pm 0.005	0.35 \pm 0.012*	1.198 \pm 0.025	0.395 \pm 0.022*
60	1.383 \pm 0.011	0.39 \pm 0.014*	1.377 \pm 0.012	0.401 \pm 0.004*
75	1.7 \pm 0.015	0.433 \pm 0.022*	1.763 \pm 0.052	0.434 \pm 0.016*
90	1.93 \pm 0.012	0.477 \pm 0.023*	1.897 \pm 0.018	0.484 \pm 0.026*
105	2.133 \pm 0.014	0.507 \pm 0.011*	2.15 \pm 0.033	0.503 \pm 0.019*
120	2.903 \pm 0.012	0.519 \pm 0.014*	2.787 \pm 0.014	0.523 \pm 0.015*
135	3.45 \pm 0.022	0.591 \pm 0.008*	3.523 \pm 0.032	0.597 \pm 0.042*
150	4.013 \pm 0.044	0.624 \pm 0.015*	4.103 \pm 0.045	0.632 \pm 0.033*
165	4.57 \pm 0.032	0.642 \pm 0.011*	4.52 \pm 0.008	0.65 \pm 0.015*
180	4.79 \pm 0.018	0.653 \pm 0.01*	4.933 \pm 0.018	0.663 \pm 0.007*

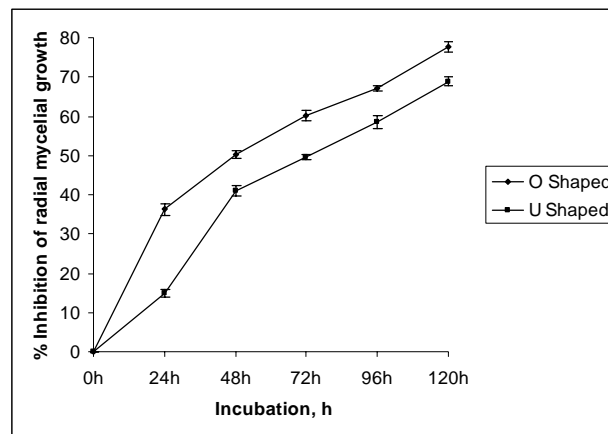


Fig 1. Inhibition of *A. alternata* by fluorescent *Pseudomonas* BRL-1 under dual plate culture using circular (O) and semicircular (U) method. Each point represents the mean \pm SE (standard error) of three separate experiments, each in triplicate.

only depends on the survivability of the biocontrol agent in the formulation it self but also on the host plant to which it is applied. Using talc based formulation of BRL-1 on *S. rebaudiana* we found that average 55% of cell population survived for 15 days after application. Lindow, (1995) suggested that *P. fluorescens* may survive on leaf surfaces by occupying particular surface sites. Fluorescent pseudomonads have been shown to colonize wheat leaves as a consequence of its foliar application (Bunster et al., 1989). Our talc-based formulation of BRL-1 applied every 15 days during the season, in two consecutive seasons successfully reduced symptoms of *Alternaria* leaf spot on *Stevia* by 82%. This ultimately helps the growers to save their crops and limits consumer exposure to hazardous fungicide. On the basis of these studies it can be concluded that the fluorescent *Pseudomonas* BRL-1 isolate has strong antagonistic properties, which might be due to the production of siderophores, proteolytic enzymes, IAA and

chitinolytic activity. It was also evident that the field application of the talc-based formulation of the antagonist considerably reduced symptoms of the disease. Global demand for medicinal plants is increasing. Scientists and industrialists are also looking for good quality products. So, outcome of the total work might help the growers to reduce the *Alternaria* leaf spot in the field thereby improving the quality and quantity of production.

Materials and methods

Organisms

The pathogen, *Alternaria alternata* (Strain Number: MAMP/C/51) isolated previously by Maiti et. al. 2007, was maintained in the potato dextrose agar medium (PDA) and stored at 4°C for further study. The biocontrol agent fluorescent *Pseudomonas* BRL-1 was obtained from our laboratory culture

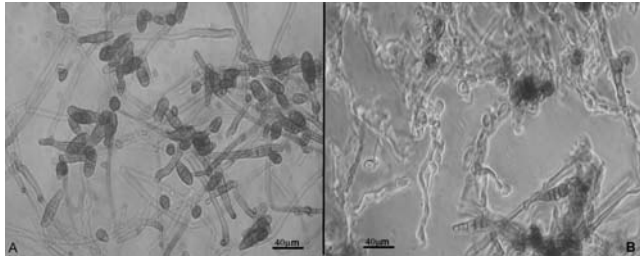


Fig. 2. A. Microscopic observation of mycelium of control plate showing normal hyphal structure and spore. B. Microscopic observation of mycelium of treated plate showing hyphal shriveling, short branching, hyphal deformities and hyphal tip swelling.

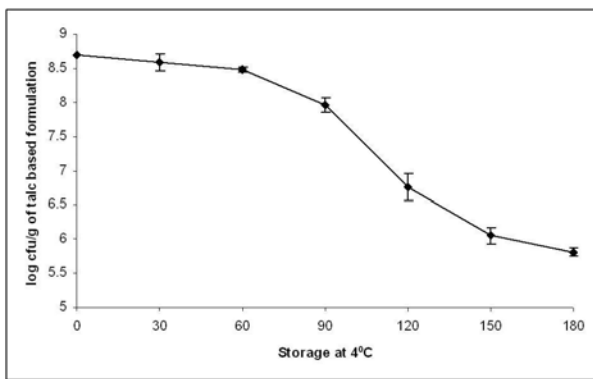


Fig. 3. Survival of fluorescent *Pseudomonas* BRL-1 in talc based formulations stored at 4°C. Each point represents the mean \pm SE (standard error) of three separate experiments, each in triplicate.

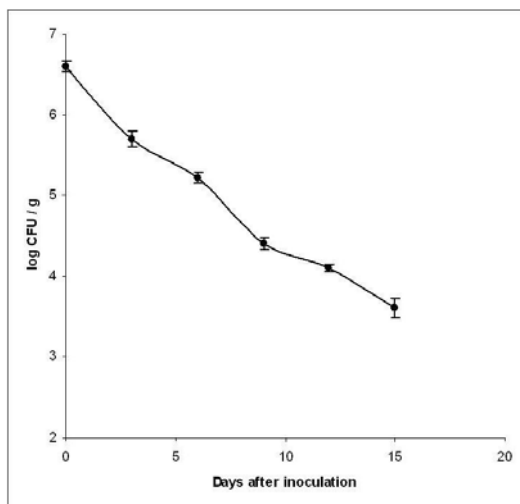


Fig. 4. Changes in the populations of BRL-1 on the phylloplane of *Stevia* growing under field condition. Each point represents the mean \pm SE (standard error) of three separate experiments, each in triplicate.

stock. The antagonist was subcultured and maintained on tryptic soy agar (TSA) medium for subsequent use.

Dual Culture Bioassay

Fluorescent *Pseudomonas* BRL-1 from 24 h old culture (10^7 cells/ml) was streaked in the peptone (1%) glucose (2%) agar (2%) (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 (Skidmore and Dickinson, 1976). 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.

Detection of in vitro antifungal compounds

Chitinase production

Fluorescent *Pseudomonas* BRL-1 was inoculated on LB plate supplemented with 0.5% colloidal chitin. Enzymatic activity was identified by the development of clear halo zones around the bacterial spots (Basha and Ulagnathan, 2002).

Detection of hydrolytic enzymes

Production of hydrolytic enzyme was qualitatively assayed in minimal medium containing gelatin, starch, pectin and carboxymethyl cellulose (CMC) for protease, amylase, pectinase and cellulase respectively (1% w/v in each case). Plates were incubated for 48 h at 30°C and formation of clear zone around bacterial colonies was read as positive (Gaur et al., 2004).

Siderophore production

Chrome Azurol S (CAS) agar medium was prepared as described by Schwyn and Neilands (1987) to detect siderophore production. CAS agar (blue agar) was inoculated at the centre of the plate with 24 h old culture of WS-1 and kept for incubation at 30°C for 72 h. The change of the blue colour of the medium to orange or presence of yellow to light orange halo surrounding the bacterial colony indicates the production of siderophore.

Production of volatile substance (HCN)

Production of volatile substance as hydrogen cyanide was tested qualitatively according the method of Wei et al. (1991). A change of colour of the filter paper strip from yellow to light brown, brown or reddish brown was recorded as weak, moderate or strong cyanogenic potential, respectively.

Indole acetic acid (IAA) production

IAA production was quantified spectrophotometrically, growing the strain in 10 mL of minimal salt media supplemented with 100 μ g / mL of tryptophan, and incubated at 30°C under shaking for 48 h. Broth culture was centrifuged at

7500 rpm for 10 min. To 1 mL of culture supernatant, 2 mL of Salkovsky reagent was added and incubated at 30°C for 25 min. Absorption was read at 530 nm and levels quantified from standard curve of IAA (Gaur et al., 2004).

Talc based formulation and survival of fluorescent *Pseudomonas BRL-1*

Talc-based formulation of the antagonist was prepared using a method developed by Vidyasekaran and Muthamilan (1995) modified. Ten gram carboxy methyl cellulose (CMC) per kg of sterile talc was used as adhesive. The bacterial suspension (8×10^9 cfu / ml) was mixed with sterile talc (400 ml / kg) and air dried (approximately to 35% w/w, moisture content). The formulation was stored at 4°C for up to 180 days and bacterial shelf life was monitored at regular interval according to Vidyasekaran and Muthamilan, (1995).

Survival of fluorescent *Pseudomonas BRL-1* on phylloplane

Survival and multiplication of fluorescent *Pseudomonas BRL-1* on the phylloplane of *Stevia* was determined following the method of Kishore et al. (2005). The talc-based formulation (4 g / l) of the antagonist was applied as foliar sprays with an initial cell density of 10^6 cfu / g of fresh leaves. Leaves were collected at 3 days intervals, washed in sterile water, serially diluted and plated (in triplicate) on King's B agar medium. Colonies of BRL-1 was determined after 48 h of incubation at 30°C utilizing its colony morphology, fluorescence characteristics. The whole experiment was performed thrice.

Field studies

Field experiments were conducted at Medicinal Plant Garden, R.K. Mission Ashrama, Narendrapur, India in 2009 and 2010 when the environmental conditions were conducive (February to July) for the rapid spread of *A. alternata* in *Stevia*. The trial was conducted as randomized complete block design with three replicate plots (3×4 m²) and forty plants per replication. 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 spot to develop naturally (Silva et al., 2004). The talc based formulation of BRL-1 was prepared by dissolving it in water (4 g / l) allowed to settle for 1 h, filtered 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. 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 where 0 = healthy leaves; 1 = small black spots on the upper surface only; 2 = spots small in size, blackish impression visible only on the lower surface; 3 = distinct spots (2 - 4) on both surfaces; 4 = numerous spots on both surfaces and 5 = leaf becoming reddish yellow and dry (Kishore et al. 2005).

Statistical analysis

The disease severity data were statistical analysed by using analysis of variance (ANOVA) followed by Duncan's Multiple

Range Test (DMRT) to find out significance level at 0.1% ($p < 0.001$)

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