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Fungal Bioagents and Botanicals Efficacy against *Alternaria alternata* Responsable for Leaf Blight Disease of *Stevia rebaudiana*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Stevia rebaudiana, a herbaceous perennial prized for its natural sweetness, has gained global acclaim and found in various parts of India including Karnataka. An exploration into leaf spot disease (caused by *Alternaria alternata* (FR.) Keissler) in Stevia was conducted under the conditions of southern Karnataka to identify effective management strategies. The symptoms initially manifested as petite circular spots of a light brown colour, subsequently evolving into irregular shapes ranging from dark brown to grey. Some spots maintained their circular form, exhibiting concentric rings or zones. Severely affected leaves exhibited the merging of numerous spots, forming expansive necrotic areas. On older leaves, concentric spots were predominantly found at the tips. The diameter of the leaf spots ranged from 2 to 18 mm. The conidial dimensions varied from 10 to 40 × 6-12 mm, displaying a mid to dark brown or olive-brown color. They were

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short-beaked, arranged in long chains, and had an oval and bean-shaped structure with 3–5 transverse septa. Considering the adverse effects of chemical fungicides, the exploration for a safer alternative to control the pathogen became a preferable option. This led to experiments involving the use of bioagents for pathogen control. The six known bioagents were evaluated by dual culture, pathogen at periphery and pathogen at the center technique to monitor antagonistic effect. The results revealed that out of all the six bioagents used, two bioagents namely *Trichoderma viride* (74.77%, 69.04% and 79.45%) and *T. harzianum* (71.25%, 59.96% and 74.78%) showed maximum growth inhibition in dual culture, pathogen at periphery and pathogen at the center methods, respectively. among the botanicals used neem (36.63%) and ginger (36.42%) found to be effective in inhibiting mycelial growth. Unraveled the strong antagonistic effect to inhibit the mycelia growth of the pathogen significantly.

Keywords: Stevia; leaf spot; Alternaria alternate; bioagent; trichoderma.

1. INTRODUCTION

Stevia [*Stevia rebaudiana* (Bertoni)] is crop of Asteraceae family. A herbaceous perennial prized for its a natural sweetener with nutritional, therapeutic and industrial importance is being used across the globe. It is native of Paraguay and South-West Brazil also called as sweet leaf, sugar leaf, sweet honey leaf and methi tulsi. Stevia leaf contains many steviol glycosides which imparts sweetness to the plant. They are mainly stevioside and rebaudioside compounds and many others. It is 100–300 times sweeter than sucrose and widely used as zero calorie sweetener all over the world on commercial scale [1]. The sweet essence of this crop is widely studied over years and its extracts are approved for safe consumption of humans without any ill effects [2]. In India, farming of stevia started because of rise in diabetic patients and demand from diabetic medicine market. At present the country's total annual production is approximately about 900 tonnes of dry leaf. The weather conditions in most parts of our country are relatively favourable for stevia farming. It is mainly grown in northern part of the country like states of Himachal Pradesh, Punjab, Uttar Pradesh, Rajasthan, Madhya Pradesh, Chhattisgarh and in some parts of Karnataka [3]. However, the cultivation of this remarkable herb is not without challenges. One of the key concerns is leaf spot disease which is due to infestation by *Alternaria alternata* (FR.) Keissler. As leaf is main site for synthesis of sweet glycosides leaf spot causes severe loss leading to ultimate yield. It is normally prevailing in all the stevia cultivating parts.

Investigation to find out suitable management strategies for alternaria leaf spot disease of stevia was carried out at department of plant pathology, GKVK, Bangalore. The pathogen affects all the above ground parts namely leaf, petiole and stem. The symptoms include small, dark brown necrotic lesion. Symptoms firstly appear as light brown small round spots. As the infection progresses, these spots became irregular and darken to shades grey to brown, while others remained round with concentrical rings. Severely affected leaves can coalesce, forming large necrotic areas. Older leaves are more prone to concentric spots, often concentrated at the tips. Under favourable condition, it results in defoliation, drying off of twig. The pathogen is both air and soil borne.

Due to hazardous effects of chemical fungicides on environment a quest for safer alternative to manage the *A. alternata* pathogen in Stevia cultivation is essential. Because of this evaluation of bioagents as potential biological control agents against *A. alternata* was under taken as they are sustainable and ecofriendly.

2. MATERIALS AND METHODS

2.1 Collection and Isolation of the Pathogen

The leaves of stevia showing typical symptoms of the leaf blight symptoms were collected from GKVK, UAS, Bengaluru. The standard tissue isolation technique was followed to isolate the pathogen in which, the stevia plants showing typical blight symptoms were collected. These leaf bits were surface sterilized with sodium hypochlorite (0.1%) solution for about one minute and washed three times serially in sterile distilled water to remove traces of sodium hypochlorite [4]. After the sterilization, leaf bits were placed in Petri plate containing potato dextrose agar (PDA) medium under aseptic condition and inoculated plates were incubated at 27±2 °C. Pure culture of the Pathogen was obtained by single spore

isolation. Dilute spore suspension was prepared; spreader on agar surface and incubated at 27±2°C; single spore was isolated and used for morphological characterization to identify the pathogen.

Composition of potato dextrose agar: Infusion from potatoes: 200 g, Dextrose: 20 g, Agar agar: 15 g and Sterile distilled water: 1000 ml.

Ready to use 39 g of potato dextrose agar was dissolved in 1000ml of distilled water. Medium was boiled to dissolve completely. Mixed solution sterilized at 1.1 kg/cm^2 pressure for 15 mins and preserved for further use.

2.2 *In vitro* Evaluation of Botanical Extracts on the Mycelial Growth of *A. alternate*

To evaluate the antifungal activity of botanicals fresh samples were washed in tap water and finally washed thrice using sterilized distilled water. They were crushed in a sterilized pestle and mortar by adding little quantity of alcohol (1:1 w/v) just enough to moisten the samples so that it was easy to crush. The extracts were strained through the two layers of muslin cloth. Finally, filtrates thus obtained from the leaves were used as stock solution [5]. To study the antifungal mechanism of plant extracts, poisoned food technique was followed as suggested by [6]. About 15-20 ml of poisoned media was poured into 90 mm Petri dishes. The 5 mm disc from 10 days old culture was taken by using an aseptic cork borer and transferred to the centre of each petri dish containing the poisoned media. the inoculated plates were incubated at 27±2°C for 12 days. Mean colony diameter in each case was recorded. The efficacy of the botanicals was expressed as per cent inhibition of mycelial growth over control which was calculated by using the formula as given by [7]. The botanicals used in this study were given in Table 1.

$$I = \frac{C-T}{C} \times 100$$

I = Per cent inhibition
C = No. of spores germinated in control
T = No. of spores germinated in treatment

2.3 *In vitro* Evaluation of Bioagents on the Mycelial Growth of *A. alternate*

Bio-agents were evaluated for their efficacy through dual culture technique. Twenty ml of sterilized and cooled potato dextrose agar medium was poured into sterilized Petri plates. Fungal antagonists were evaluated by inoculating a pathogen at one side of the Petri plate and the antagonist at exactly opposite side of the same plate by leaving about 4 cm gaps [8]. For this, actively growing cultures were used. After required period of incubation i.e., when the

Table 1. Botanical and common name of species

Sl. No.	Botanical name	Common name	Plant parts used
1	*Azadirachta indica*	Neem	Leaves
2	*Bougainvillea spectabilis*	Bougainvillea	Leaves
3	*Lantana camara* L.	Lantana	Leaves
4	*Psidium guajava*	Guava	Leaves
5	*Allium sativum* L.	Garlic	Bulb
6	*Moringa oleifera*	Moringa	Leaves
7	*Cymbopogan ambiguous*	Lemon grass	Leaves
8	*Allium cepa* L.	Onion	Bulb
9	*Zingiberis officinalis*	Ginger	Rhizome

Table 2. List of fungal bio-agents evaluated against *A. alternate*

Sl. No.	Bio-agents	Isolates	Source
1	*Trichoderma viride*	TV	Department of Microbiology,GKVK
2	*Trichoderma viride*	TV-2	Department of Plant Pathology,GKVK
3	*Trichoderma viride*	TV-3	Department of Plant Pathology,GKVK
4	*Trichoderma harzianum*	Th-41	Department of Plant Pathology,GKVK
5	*Trichoderma harzianum*	Th-44	Department of Plant Pathology,GKVK
6	*Trichoderma harzianum*	Th	Department of Plant Pathology,Chintamani

growth in control plate recorded 90 mm in diameter [9], the radial growth of the pathogens was measured. Per cent inhibition over control was worked out according to the equation given by [7]. The six different fungal antagonistic organisms were used against stevia leaf spot pathogens was given in Table 2.

3. RESULTS AND DISCUSSION

3.1 Collection and Isolation of the Pathogen

In culture, the fungal colony was initially white, cottony with profuse aerial mycelium which gradually turned greenish grey. Aged culture appeared completely brown with no aerial mycelium. The microscopic studies of the isolated fungus revealed that conidia of the pathogen were three - four celled, Conidiophores short to long, simple or branched arising singly. Conidiophores were hyaline to golden brown coloured. Conidia are typically muriform, dark brown, thick walled, in long chains. Based on the characters of colony and morphological characters of conidiophores and conidia, the fungus was identified as *A. alternata* [10-12].

3.2 *In vitro* Evaluation of Botanicals against *A. alternata*

The study was carried out to know the antifungal activity nature of different plant extracts against *A.alternata* by poison food technique. Based on the observation of radial growth of the fungus, the per cent inhibition was calculated. The effectiveness of different plant extracts in reducing the mycelial growth of *A.alternata* varied greatly noted in Fig. 1.

The results presented in Table 3 and Fig. 1 revealed statistical difference between plants extract per cent inhibition at three different concentrations with three replications. Neem (36.63**%**) was found to be most effective and statistically on par with Ginger (36.42%) and lemon grass (36.41%). The least inhibition of mycelial growth was observed in Moringa (18.76%).

The findings are in collaboration with the earlier findings [13] reported that plant extract of *Azadirachta indica* exhibited maximum mycelial inhibition of 80.53 mm at 15 per cent concentration, whereas *Allium sativum* showed 21.60 mm of mycelial inhibition which was significantly lower over rest of plant extracts.

The efficacy of botanicals against *A. solani* was assessed by [14] using the poisoned food technique at a 10 percent concentration. In the crude extraction method, *A. indica* demonstrated the highest inhibition at 60.49%, followed by Zingiber officinale at 54.73% and Ocimum sanctum at 53.09%. Meanwhile, the acetone extraction method exhibited a slight increase in the inhibition of *A. solani*'s mycelial growth, with A. indica leading at 64.24%, followed by *O. sanctum* at 58.62% and *Z. officinale* at 57.32%.

Table 3. *In vitro* evaluation of botanicals against *A. alternate*

Sl. No.	Botanicals	Per cent inhibition over control			
		Concentration (%)			
		10 %	**15 %**	**20 %**	**Mean**
1	Ginger	23.33 (28.87) *	38.89 (38.56) *	47.04 (43.28) *	36.42 (33.09) *
2	Garlic	22.22 (28.11)*	32.22 (34.57)*	35.56 (36.59) *	30.00 (33.09) *
3	Lantana	18.89 (25.75) *	30.00 (33.20)*	34.44 (35.92)*	27.77 (31.62)*
4	Guava	16.67 (24.09)*	22.22 (28.11)*	31.11 (33.89)*	23.33 (28.69)*
5	Onion	28.89 (32.50) *	32.59 (34.80)*	38.89 (38.56)*	33.44 (35.28)*
6	Neem	30.37(33.43)*	36.67 (37.25)*	40.00 (39.22)*	36.63 (36.63)*
7	Moringa	8.52 (16.96)*	19.63 (26.29)*	28.15 (32.03)*	18.76 (25.09)*
8	Lemon grass	32.22 (34.57)*	34.81 (36.15)*	42.22 (40.51)*	36.41 (37.07)*
9	Bougainvillea	10.00 (18.43)*	21.85 (27.86)*	27.78 (31.79)*	19.87 (26.02)*
Mean		21.23 (26.96)*	29.87 (32.97)*	36.13 (36.86)*	29.18 (31.84)*
		Botanicals (B)	**Concentration(C)**		**Interaction(B×C)**
S.Em ±		0.22	0.12		0.40
CD @ 1 %		0.86	0.50		1.50

Fig. 1. *In vitro* evaluation of botanicals against *A. alternata*
T_0: Control,T_1: Ginger, T_2: Garlic, T_3: Lantana,T_4: Guava, T_5: Onion, T_6: Neem, T_7: Moringa, T_8: Lemon grass, T_9: Bougainvillea

3.3 Evaluating the Efficacy of *Trichoderma* spp. as Bio-Agents

Six known bioagents were tested for their efficacy in controlling *A. alternata* through dual culture, pathogen at the periphery, and pathogen at the center techniques. The results were promising, with two bioagents, *Trichoderma viride* (TV) (79.45 %) was found highly superior in inhibiting the mycelial growth followed by *Trichoderma harzianum* (TH) (74.78 %). The *T. viridae* 2 (TV2), demonstrating the highest levels of growth inhibition in dual culture which was on par with *Trichoderma harzianum* (TH). The least inhibition was recorded in *T. harzianum*-41 (56.96%). These findings unveil the strong antagonistic effect of *Trichoderma* spp., significantly inhibiting the mycelial growth of the *A. alternata* pathogen (Table 4 and Fig. 2).

The results align with the prior discoveries of the study conducted by [15]. All seven fungal and two bacterial antagonists demonstrated noteworthy inhibition of mycelial growth in *A. alternata*. Notably, *T. viride* exhibited the highest mycelial growth inhibition at 86.85%, followed by *T. hamatum* at 82.04% and *A. niger* at 81.11%.

Table 4. *In vitro* evaluation of bioagents against *A.alternata*

Sl. No.	Fungal bioagent	Per cent inhibition over control*
1	*T. harzianum*-41 (TH-41)	56.96 (48.98)*
2	*T. harzianum* -44 (TH-44)	71.25(57.55)*
3	*T. harzianum* (TH)	74.78 (59.83)*
4	*Trichoderma viride* 2 (TV2)	74.77 (59.82)*
5	*Trichoderma viride* -3 (TV3)	69.45 (56.42)*
6	*Trichoderma viride* (TV)	79.45 (63.02)*
SEm ±		0.46
CD @ 1%		1.98

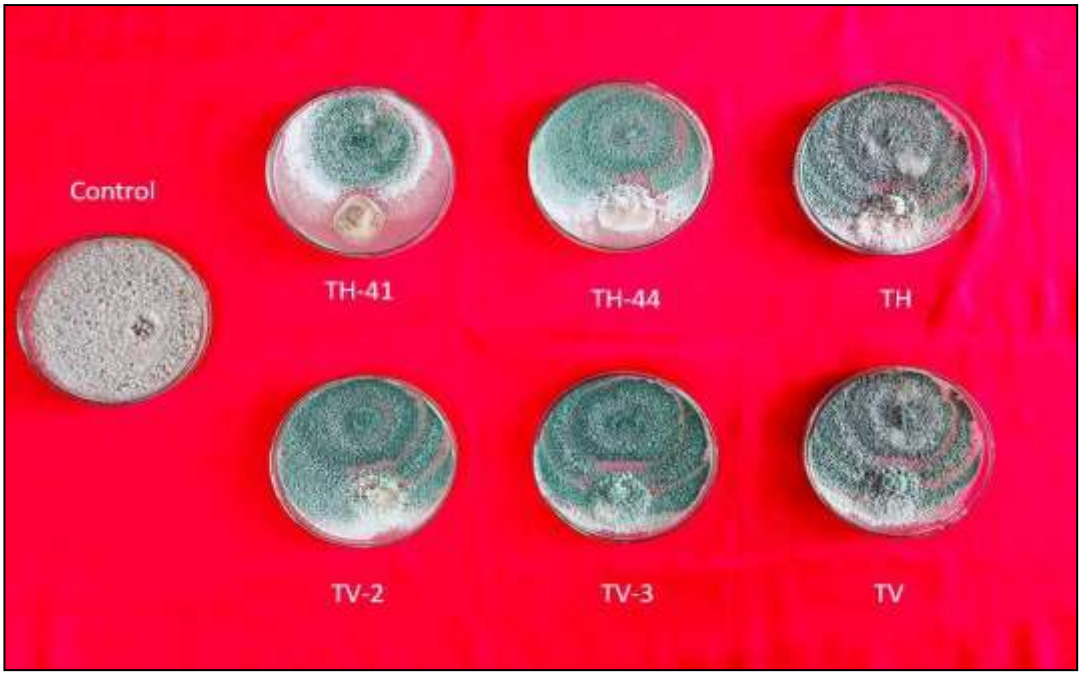

Fig. 2. *In vitro* evaluation of fungal bio agents against *A. alternate*

4. CONCLUSION

The prevalence of *A. alternata*-induced leaf spot disease poses a significant threat to Stevia cultivation in South Karnataka. However, this study's findings offer hope for effective and sustainable management strategies by harnessing the biological control potential of *T. viride* and *T. harzianum*. In this study the *T. viridae* (TV) and *T. harzianum* has shown 79.45 and 74.78 % inhibition respectively. Among the botanicals neem and ginger were found promising with 36.63 and 36.42 % inhibition respectively. By reducing the reliance on chemical fungicides and embracing bioagents, Stevia cultivators can mitigate the impact of leaf spot disease and promote a healthier and more environmentally friendly agricultural ecosystem in the region. Further research and implementation of these biological control methods hold promise for the future of Stevia production in South Karnataka.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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