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Evaluation of botanical extracts and bioagents under *in vitro* for the management of betelvine wilt caused by *Sclerotium rolfsii* Sacc.

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Abstract
The soil-borne pathogen *Sclerotium rolfsii* Sacc., which causes wilt disease, is one of the diseases that most frequently infest betelvine (*Piper betle* Linn.). The goal of the current study was to assess the inhibitory effects of four antagonistic biocontrol agents and aqueous extract of eight different plant species of plants under *in vitro* condition for their inhibitory effect on mycelial growth of *S. rolfsii*. Among the plant extracts tested at three different concentrations, 15 per cent concentration of all plant extracts were significantly found superior to 5 and 10 per cent. At 15 per cent concentration of plant extracts, maximum 62.19 per cent inhibition of mycelial growth was recorded in tulsi leaf extract followed by marigold leaf extract (57.11%). Among the biocontrol agents tested *Trichoderma harzianum* I showed maximum antagonistic effect and found to be significantly superior in inhibiting the mycelial growth of *S. rolfsii* (62.64%).

Keywords: Betelvine, Biocontrol agents, *in vitro*, Mycelia, Plant extracts, *Sclerotium rolfsii*

1. Introduction
Betelvine (*Piper betle* Linn.) is a perennial, shade loving ever green creeper belongs to the family Piperaceae. It is commercially cultivated in many parts of the world especially in the tropical and sub-tropical countries. Every part of the vine has high medicinal value, the presence of phenolic compound hydroxyl-chavicol, with anti-carcinogenic property has also been identified in betel leaves [1].

Betelvine wilt caused by *Sclerotium rolfsii* Sacc. is one of the most destructive disease of betelvine. All stages of vines are susceptible to the disease. Usually, the infection begins at the collar. On the stem and roots, one can discern whitish cottony mycelium. The stem section exhibits tissue decay at the site of the attack, and the plants exhibit leaf drop and wilting before drying out completely. The extent of losses varies from 5-90 percent [2, 3]. The fungus can overwinter as mycelium in infected tissues or plant debris or as sclerotia near soil surface or buried in soil which serve as a major source of primary infection by germinating in response to alcohols and other volatile compounds released from decomposing plant material [4].

Currently, the disease is primarily controlled by the application of agrochemicals like fungicides. However, the uncontrolled use of pesticides disrupts the natural ecological balance by destroying both antagonistic and/or beneficial microbes. As a cost-effective, long-lasting, and environmentally safe alternative to chemicals, plant extracts, biocontrol agents, and soil amendment materials, among others, suppress the pathogen and protect the plants [5]. They also prevent the emergence of pathogen strains with increased resistance. Therefore, the goal of the current study was to evaluate the efficacy of botanical extracts and biocontrol agents in an *in vitro* condition in order to create an effective management strategy that incorporates both chemical and non-chemical approaches to the disease.

Material and Methods
To assess the growth inhibition and antagonistic properties of plant extracts and biocontrol agents respectively, *in vitro* experiment was carried out in the plant pathology laboratory of UAHS, Shivamogga.

***In vitro* evaluation of plant extracts**

Fresh samples of the following widely accessible plant materials were gathered and used for extraction: tulsi leaves, marigold leaves, neem leaves, noni leaves, and turmeric rhizomes. These plant materials were extracted using hot water on a w/v (100 g/100 ml) ratio, and the concentrate was kept in the refrigerator for later use. The plant extracts' antifungal efficacy was examined using poisoned food technique. To achieve 5, 10, and 15% concentrations, the desired amount of the concentrate was combined with sterilized and cooled Potato dextrose agar medium at the time of pouring. Twenty ml of the medium was poured into petriplate, mycelial disc of the fungus was placed at the centre of the petriplate and were replicated thrice. The per cent inhibition over control was worked out according to equation given by Vincent [6].

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

Where,
I = Per cent inhibition
C = Radial growth in control
T = Radial growth in treatment

***In vitro* evaluation of bio-agents**

The efficacy of four antagonistic bio-agents *viz*., *Trichoderma harzianum* (Rifai) I (UAS Dharwad isolate), *Trichoderma harzianum* (Rifai) II (UAHS Shivamogga isolate), *Pseudomonas fluorescens* (Flugge) Migula and *Bacillus subtilis* (Ehrenberg) Cohn were obtained from Department of Plant Pathology, UAHS, Shimoga and were tested against *S. rolfsii* for radial growth inhibition on the PDA media using through dual culture technique under *in vitro* condition.

For this study both bio-agents and test fungus were cultured on potato dextrose agar in order to get fresh and active growth of fungus. Twenty ml of sterilized and cooled Potato dextrose agar was poured into sterilized petriplates. Fungal antagonists were evaluated by inoculating the pathogen at one side and the antagonist exactly opposite side to it in the same petriplate by leaving 3-4 cm gap. For this, actively growing culture was used. In case of bacterial antagonist evaluation, two mycelial discs of pathogen were inoculated and bacterial antagonist was streaked in the centre of the plate. The plates were replicated five times. After required period of incubation *i.e.* after control plate reached 90 mm diameter, the radial growth of pathogen was measured. Per cent inhibition over control was calculated as described above.

Results and Discussion

Efficacy of plant extracts extracts and bioagents was studied under *in vitro* condition by following dual culture method and the results obtained are presented under the following heads with relevant discussion.

In vitro* evaluation of plant extracts extracts against *S. rolfsii

The effect of plant extracts on the per cent inhibition of mycelial growth of *S. rolfsii* at three concentrations differed significantly. Among the eight plant extracts tested, maximum of per cent inhibition of mycelial growth (40.06%) was recorded in tulsi leaf extract which was significantly superior to all other tested plant extracts, followed by marigold leaf extract (37.81%), neem leaf extract (27.43%), turmeric rhizome extract (27.26%), noni leaf extract (25.80%), mehandi leaf extract (24.49%) and garlic bulb extract (24.14%). Least inhibition was recorded in eucalyptus leaf extract (21.37%). Among the tested three concentrations, 15 per cent concentration of all plant extracts was significantly found superior to 5 and 10 per cent. At 15 per cent concentration of plant extracts, maximum of 62.19 per cent inhibition of mycelial growth was recorded in tulsi leaf extract followed by marigold leaf extract (57.11%) and turmeric rhizome extract (53.44%). Further neem leaf extract, noni leaf extract, mehandi leaf extract, garlic bulb extract and eucalyptus leaf extract showed 50.59, 49.78, 47.89, 43.33 and 36.48 per cent inhibition respectively (table 1).

The findings are in line with the findings of Muthukumar [7] reported the efficacy of 16 essential oils against mycelia growth of *S. rolfsii*, among these citronella, lemongrass, tulsi and turmeric oils found to be effective at 1.0 per cent concentration and caused complete growth inhibition of pathogen. Begum *et al.* [8] found that, among botanicals tested at 5 and 10 per cent concentrations, significantly highest average inhibition was recorded with neem (74.81%), followed by tulsi (67.10%) and nirgudi (65.81%). The antifungal property of tulsi is due to phenolic compound such as eugenol, it is related to its lipophilic character in that they increase the fluidity and permeability of the cell membrane of microorganisms [9].

Table 1: Effect of different plant extracts on mycelial growth of *S. rolfsii*

Plant extracts	Common name	Plant part used	Per cent inhibition of mycelia growth			
			Concentrations (%)			Mean
			5	10	15	
Allium sativum L.	Garlic	Bulb	0.00 (0.00)*	29.07 (32.65)	43.33 (41.19)	24.14
Curcuma longa L.	Turmeric	Rhizome	0.00 (0.00)	28.33 (32.18)	53.44 (47.00)	27.26
Morinda citrifolia L.	Noni	Leaves	0.00 (0.00)	27.63 (31.73)	49.78 (44.90)	25.80
Eucalyptus tereticornis L.	Nilgiri	Leaves	0.00 (0.00)	27.63 (31.73)	36.48 (37.18)	21.37
Lawsonia inermis L.	Mehandi	Leaves	2.59 (9.27)	23.00 (28.67)	47.89 (43.81)	24.49
Ocimum sanctum L.	Tulsi	Leaves	6.44 (14.71)	51.56 (45.91)	62.19 (52.08)	40.06
Azadirachta indica Juss.	Neem	Leaves	3.52 (10.82)	28.19 (32.08)	50.59 (45.36)	27.43
Tagetes erecta L.	Marigold	Leaves	9.89 (18.34)	46.44 (42.98)	57.11 (49.11)	37.81
Control			0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
	S.Em±			CD at 1%		
Plant extracts (B)	0.38			1.031		
Concentration (C)	0.24			0.634		
B x C	0.67			1.796		

* Figures in parenthesis are arcsine transformed values

In vitro* evaluation of biocontrol agents against *S. rolfsii

There were significant differences among all the tested bio-agents as presented in table 2. *Trichoderma harzianum* I was found to be significantly superior in inhibiting the mycelial growth of *S. rolfsii* (62.64%) followed by *Trichoderma harzianum* II (57.08%). There was no inhibition of mycelial growth of fungus in *Pseudomonas fluorescens* and *Bacillus subtilis* (0.00%).

Table 2: *In vitro* evaluation of bioagents against *S. rolfsii*

Bio agent	Per cent inhibition of mycelial growth
Trichoderma harzianum I	62.64# (52.35)*
Trichoderma harzianum II	57.08 (49.10)
Bacillus subtilis	0.00 (0.00)
Pseudomonas fluorescens	0.00 (0.00)
S.Em±	0.10
CD at 1%	0.43

* Figures in parenthesis are arcsine transformed values
# mean of five replications

Similar types of observations were made by Basamma [10] and Kulkarni [11] who noticed 59.81 and 53.33 per cent inhibition of mycelial growth of *S. rolfsii* by *T. harzianum* these results indicated that *Trichoderma* isolates have competition, mycoparasitic and lysis effect on the pathogen. It may be due to production of antibiotic substance such as gliotoxin, viridin and some cell wall degrading enzymes which might have diffused air filled pores, which are detrimental to the growth of *S. rolfsii* as reported by Brain [12] and also certain biologically active heat stable metabolites such as ethyl acetate [13]. These results are also in agreement with results of Karthikeyan [14] and Mukharjee *et al*. [15]. Showed the inhibition of mycelial growth of *S. rolfsii* by *T. harzianum* is due to the penetration of the antagonist hyphae into hyphae of pathogen at the place of contact. The bioagents used in the present study are easily producible, biodegradable, less expensive and cause no environmental hazards to human health. These are ecologically safe and culturally more acceptable among the farmers.

By reducing cultivation costs and avoiding health risks, the use of botanicals and bio-agents offers an alternative to the use of synthetic pesticides. The *in vitro* results indicate that there are actually substitutes for synthetic fungicides that can be used to manage this infamous soil-borne fungus: *S. rolfsii*, which costs millions of dollars in losses. Combining both strategies (the use of plant extracts and antagonistic microbes) may result in the creation of a crop production system that is commercially viable.

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