Research Article:

IN VITRO AND *IN VIVO* EVALUATION OF ESSENTIAL OILS AGAINST *Sclerotium rolfsii* Sacc.

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ABSTRACT

Sclerotium rolfsii Sacc., the causal agent of foot rot disease in *Capsicum frustescens* L. (chilli), is a soil-borne necrotrophic fungal pathogen that leads to significant economic losses. The pathogen's broad host range and ability to form persistent sclerotia have made it challenging to manage. As an alternative to conventional approaches, botanical and essential oils have been investigated for their disease management potential. In this study, five essential oils were tested *in vitro* at different concentrations against the pathogen, and only three were choosen for further evaluation in an *in vivo* experiment on chilli seedlings. The laboratory tests showed that palmarosa and lemon grass completely inhibited mycelial growth of the pathogen at 500 ppm, and at the same concentration, palmarosa, lemon grass, and calamus completely inhibited sclerotial germination. However, none of the tested essential oils were effective against the pathogen in the *in vivo* test. Despite of having no efficacy in planta assays, the study concluded that the effective essential oils identified *in vitro* have strong antifungal activity and could be used as an alternative to harmful fungicide. However, more work are warranted for developing proper formulation to maintain their chemical composition in order to manage the disease *in vivo* conditions.

Key words: Chilli, essential oil, sclerotia, *Sclerotium rolfsii*

INTRODUCTION

Sclerotium rolfsii Sacc. (Teleomorph *Athelia rolfsii* (Curzi) C. C. Tu & Kimbr.) is a soil-borne pathogen of aggressive nature and causes considerable damage to young seedlings. It occurs in diverse soils, has a wide host range and worldwide distribution (Bhagat, 2014). The pathogen causes a great economic loss on various crops such as groundnut, tomato, brinjal, pepper, chilli, potato, sunflower, cotton, beans, pea, maize, wheat, cucurbits, crucifers with 25-50 percent seedling mortality having yield depletion ranging from 10-60 percent in different field conditions (El-Nagar et al., 2013). *Sclerotium rolfsii* primarily attacks host stems near the soil-line, but has the potential to infect any part of a plant under favourable environmental conditions (Agrios, 2005). Susceptible plant tissues, lower stems, roots, and fruit can be directly penetrated by contacting hyphae under the conditions of warm and wet weather. During the infection process, the pathogen secretes oxalic acid and endopolygalacturonase, which degrade plant tissues and cells, and eventually lead to decay (Favaron et al., 2004; Punja, 1985).

Paparu et al. (2020) reported the mycelium of *S. rolfsii* is white, fluffy, usually growing in strands and in radial fans producing asexual reproductive propagules, sclerotia, compact hyphal aggregates (size of mustard seed). A wide range of host, profuse mycelial growth, and ability of the pathogen to produce persistent sclerotia has made its management more challenging (Asad

& Khanum, 2007). Various measures like using healthy seedling, improving farming practices, use of resistant crop varieties, biological and botanical measures, chemicals and integrated disease management are strategically useful for the management of the diseases caused by *S. rolfsii* (El-Nagar et al., 2013).

In recent days, various botanicals and essential oils are also being used for the management of fungal and bacterial infection. Essential oils (EOs) represent a new class of crop protectants due to their effects, short shelf-life and low toxicity to the environment (Arraiza et al., 2018). Compounds such as carvacrol, thymol, linalool, cymene, pinene are known to exhibit anti-microbial activity (Derbalah et al., 2012). These are the major components of essential oils with promising anti- fungal applications (Derbalah et al., 2012). The management of soil borne crop diseases by the application of chemical pesticides are not effective for long-term use due to concerns of expense, exposure risks, residues and other health and environmental hazards. Moreover, the potential for the development of resistance towards synthetic fungicides in pathogenic fungi is of great concern. The present study was conducted to identify potential essential oils effective in inhibiting the growth and development of *S. rolfsii*.

MATERIALS AND METHODS

Isolation of *Sclerotium rolfsii*

The pathogen was isolated from infected collar region of Chilli crop collected from a field at Fulbari, Chitwan. The collar region was washed and cut into small pieces. The infected samples were surface sterilized with one per cent sodium hypochlorite (NaOCl) for one minute followed by triple rinsing with distilled water. The samples were kept in the Petri plates with moist two layers of blotter papers. The plates were incubated at 25 ± 1 °C in an incubator until sclerotia were produced and the sclerotia produced were transferred to potato dextrose agar (PDA) to obtain pure culture. The pure culture was maintained at 4 °C.

In vitro evaluation of essential oils against *S. rolfsii*

The essential oils selected for the experiment were wintergreen (*Gaultheria fragrantissima*), calamus (*Acorus calamus*), zanthoxylum (*Zanthoxylum armatum*), palmarosa (*Cymbopogon martinii*), and lemongrass (*Cymbopogan flexuosus*). These essential oils were obtained from National Organics, Kathmandu, Nepal which were commercially extracted by steam distillation method. Poisoned food technique as described by Euloge et al. (2012) was used for the evaluation of antifungal activity of different essential oils against *S. rolfsii*. The experiment was conducted in two factorial completely randomized design with five replications. Potato dextrose agar (PDA) medium containing three different concentrations of five essential oils was prepared in Erlenmeyer flask by adding 0.01 ml, 0.05 ml and 0.10 ml of 100 percent pure essential oils in 100 ml melted PDA medium for 100 ppm, 500 ppm, and 1000 ppm respectively using micropipette. The Erlenmeyer flask was rotated manually for uniform mixing of the oil with media. About 20 ml of poisoned media was poured into Petri plates of 9 cm diameter one day before inoculation under the aseptic conditions. Fresh culture of the pathogen was grown on PDA medium from pure culture in BOD incubator at 25 ± 1 °C five days before setting up the experiment. Similarly, the subculture of the pathogen prepared from pure culture in BOD incubator at 25 ± 1 °C was allowed to grow for 14 days to get matured sclerotia. Mycelial disc (5 mm diameter) from the actively growing margin of 5 days old culture of the pathogen and single matured sclerotium from 14 days old culture of the pathogen was inoculated at the center of the Petri plates containing poisoned media of different concentrations as mentioned earlier. Control plates without essential oils were also inoculated by pathogen mycelium and sclerotium.

The inoculated plates were incubated in BOD incubator at 25 ± 1 °C. The plates inoculated with sclerotia were observed for sclerotial germination in every 24 hours after inoculation until there was full growth of the pathogen after sclerotial germination in the control plate. Collapse of the sclerotial rind with the eruption of hyphae (mycelial strands) from the pathogen's hard, survival structure (sclerotia) indicated sclerotial germination. Sclerotial germination was measured qualitatively through eye observation and colony growth was measured in those treatments in which sclerotia germinated. Similarly, the plates inoculated with mycelial disc were observed for the colony growth of the pathogen. Colony diameter of the pathogen was measured from the underside of the plate in two directions in every 24 hours after inoculation until there was full growth of the pathogen in the control plates and the average diameter was recorded. The percent growth inhibition was calculated using the radial growth measurement of the test pathogen by using the formula given by Vincent (1947).

$$P_i(\%) = \frac{C - T}{C} \times 100\%$$

Where, P_i = Percent growth inhibition

C = Radial growth of the test pathogen in control

T = Radial growth of the test pathogen in treatment

In vivo* evaluation of essential oils against *S. rolfsii

Based on the *in vitro* results, only three essential oils (palmarosa, calamus and lemon grass) were selected for *in vivo* evaluation. For this, mass culture of the pathogen was prepared in the sorghum grain. Sorghum was soaked overnight in water and dried in shade area in tray to drain excess water from the grain. Two hundred gram of the soaked and dried sorghum grains were filled in autoclavable polybags. The polybags filled with the grains of sorghum were autoclaved at 121 °C temperature and 15 psi pressure for 20 minutes on two consecutive days and allowed to cool under aseptic condition. The substrate in polybag was inoculated with 5 mm diameter mycelial disc (2 discs per polybag) from the actively growing margin of 5 days old culture of *S. rolfsii* and incubated for 12 days at 25 ± 1 °C.

NS-1701 variety of chilli was selected for the *in vivo* experiment where seedlings were raised in plugmix media in seedling raising tray. The pot with 1 kg capacity was filled with sterilized soil. Three different essential oils were diluted in water in separate glass jar by mixing 2 ml of essential oils into 1000 ml of water to prepare the final concentration of 2000 ppm and vigorously shaked. The chilli seedlings having 2-3 true leaves were uprooted and dipped into the diluted three essential oils for 10 minutes. Similarly, a control was kept where the seedling was dipped in distilled water only. Single treated chilli seedling was transplanted in each pot and again drenched with 10 ml prepared solution of essential oils (Madhavi & Bhattiprolu, 2011). There were five seedlings per experimental unit. The pots were arranged in completely randomized design with five replications in the screen house. The temperature in the screen house ranged from 27-30 °C with 60-70% relative humidity during the experimental period.
One week after transplanting, the seedlings already treated with essential oils were inoculated with two gram mass culture of *S. rolfsii* at the surface of soil and thoroughly mixed with upper 2 inch soil layer of the pot (Madhavi & Bhattiprolu, 2011). The disease incidence was calculated using the following formula (Cooke, 2006).

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected seedlings}}{\text{Total number of seedlings observed}} \times 100\%$$

Statistical analysis

The data recorded under *in vitro* and *in vivo* tests were tabulated in Microsoft Excel 2007 data worksheets. Gen Stat 15th edition computer program was used for the analysis of variance

(ANOVA) and for data with significant difference, mean comparison was done by Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

In vitro evaluation of essential oils against mycelial growth of *S. rolfsii*

Essential oils of palmarosa and lemongrass were statistically highly significant and most effective against the pathogen as they completely inhibited the colony growth at 500 ppm concentration (Table 1). It was followed by calamus by recording 86.98 percent growth inhibition at 1000 ppm concentration. At 500 ppm concentration, least percent growth inhibition (5.02 %) was recorded by wintergreen followed by zanthoxylum and calamus which were statistically at par with each other by recording 39.67 and 37.08 percent growth inhibition respectively. Wintergreen proved least effective as it recorded only 39.82 percent growth inhibition at higher concentration of 1000 ppm.

Table 1. ***In vitro*** **efficacy of essential oils at different concentrations on per cent mycelial growth inhibition of** ***S. rolfsii***

Essential oil	Mycelial growth inhibition (%)			Mean
	100 ppm	500 ppm	1000 ppm	
Wintergreen	-0.21^{f}	5.02^{f}	39.82^{e}	14.87^{e}
Calamus	6.05^{f}	37.08^{e}	86.98^{b}	43.37^{c}
Zanthoxylum	3.34^{f}	39.67^{e}	61.93^{c}	34.98^{d}
Palmarosa	68.40^{c}	100^{a}	100^{a}	89.47^{a}
Lemongrass	51.84^{d}	100^{a}	100^{a}	83.95^{b}
Mean	25.89	56.35	77.75	
	Essential oil	Concentration	Essential oil x Concentration	
F-test	***	***	***	
LSD (≤0.05)	4.55	3.53	7.89	
S.Em (±)	1.59	1.24	2.77	
CV (%)	10.4	10.4	10.4	

LSD, Least significant difference; S.Em (±), Standard error of mean; CV%, Coefficient of variation. Means followed by common letter(s) within column are not significantly different among each other based on DMRT at 0.05 level of significance

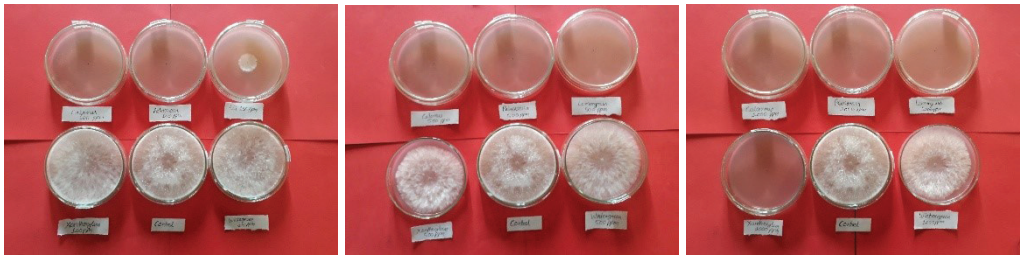
In vitro evaluation of essential oils against sclerotial germination and radial growth of *S. rolfsii*

The sclerotial germination of *S. rolfsii* was completely inhibited in the plates amended with 100 ppm calamus and palmarosa oils (Table 2). The plates amended with 100 ppm lemon grass also recorded only 2.32 cm radial colony growth after sclerotial germination which was significantly lower compared to non-amended control plates. Complete inhibition of sclerotial germination was also found in the plates amended with 500 ppm lemon grass oil. At higher concentration of 1000 ppm, essential oils of calamus, zanthoxylum, palmarosa, and lemongrass were statistically highly significant and at par with each other as there was no or very negligible amount of sclerotial germination. The plates amended with 1000 ppm wintergreen oil had sclerotial germination along with radial colony growth of 7.76 cm of the pathogen. The result showed that wintergreen was least effective against the pathogen among other tested essential oils (Table 2). The result of this experiment was further illustrated by Fig. 1.

Table 2. Sclerotial germination and radial colony growth (cm) of *S. rolfsii* as affected by different concentrations of essential oils

Essential oil	Radial colony growth (cm)			Mean
	100 ppm	500 ppm	1000 ppm	
Wintergreen	9[a]	8.98[a]	7.76[b]	8.58[b]
Calamus	0[e]	0[e]	0[e]	0[e]
Zanthoxylum	9[a]	7.33[c]	0.15[e]	5.49[c]
Palmarosa	0[e]	0[e]	0[e]	0[e]
Lemongrass	2.32[d]	0[e]	0[e]	0.77[d]
Mean	4.06	3.26	1.58	
Control (water)	9			
	Essential oil	Concentration	Essential oil x Concentration	
F-test	***	***	***	
LSD (≤0.05)	1.67	1.29	2.89	
S.Em (±)	0.59	0.46	1.02	
CV (%)	3.4	3.4	3.4	

LSD, Least significant difference; S.Em (±), Standard error of mean; CV%, Coefficient of variation. Means followed by common letter(s) within column are not significantly different among each other based on DMRT at 0.05 level of significance



100 ppm 500 ppm 1000 ppm

Fig. 1. Growth of *Sclerotium rolfsii* in different concentrations of essential oils at Rampur, Chitwan

In vivo* evaluation of different essential oils against *S. rolfsii

Essential oils of palmarosa, lemongrass, and calamus were found to be ineffective against the pathogen as there was 100 percent disease incidence in the chilli seedlings treated with essential oils similar to that of control even if they performed very well *in vitro* (Table 3 & Fig. 2). However, the time of first symptoms appearance in the treated seedlings was slightly longer by 2-3 days compared to untreated seedlings.

Table 3. Effect of essential oils on foot rot incidence of chilli seedlings caused by *S. rolfsii* at Rampur, Chitwan, 2020-21

Treatments	Disease incidence (%)
Palmarosa	100
Lemongrass	100
Calamus	100
Control (water)	100

Fig. 2. Experimental pots with chilli seedlings for *in vivo* evaluation of essential oils against *Sclerotium rolfsii* at Rampur, Chitwan

Among the various diseases responsible for the significant loss of higher productivity of chili (*Capsicum frutescens* L.), foot rot or collar rot of chilli caused by necrotrophic soil-borne fungal pathogen *S. rolfsii* is also one of the economic disease of chilli (Baidya, 2018). As most of the losses due to *S. rolfsii* are incurred during the seedling stage, it would greatly affect the initial plant stand of transplanted chilli seedlings. Being soil-borne pathogen, most of the management practices need to be done before transplanting of chilli seedlings in order to prevent the foot rot and wilt disease (Remesal et al., 2010).

Five different essentials oils at three levels of concentrations were evaluated *in vitro* against *S. rolfsii*. Among them, palmarosa and lemon grass completely inhibited the mycelial growth of the pathogen even at concentration of 500 ppm while palmarosa, lemon grass, and calamus completely inhibited the sclerotial germination at 500 ppm. Overall results showed that the essential oils of palmarosa, lemon grass, and calamus were effective in inhibiting the sclerotial germination and colony growth of the pathogen. Wilson et al. (1997) found that the limonene and β−myrcene were the major chemical constituents behind the antifungal activity of the essential oils of lemon grass and palmarosa respectively. β-Asarone (83.2%) and α-asarone (9.7%) were the major constituents in the rhizome oil of *Acorus calamus* having high antifungal activity (Raina et al., 2003). Sharma et al. (2009) found the most significant antifungal activity of essential oils extracted from *Acorus calamus*, *Curcuma longa*, *Pimpinella anisum* and *Vetiveria zizanioides* against *Sclerotium rolfsii* and *Rhiozoctonia bataticola*. Ali & Yousef (2013) found that the essential oils of *Cymbopogon citratus* caused morphological changes of fungal hyphae inhibiting fungal viability and spore germination. Bansod and Rai (2008) also reported higher antimycotic activity by the essential oils of *Cymbopogon martini* and *C. citratus* among the other 15 essential oils from various medicinal plants. Microscopic observation on the effect of β-Asarone found in essential oil of *Acorus calamus* on fungal hyphae and conidia showed drastic morphological alterations with shrunken and collapsed form (Phongpaichit et al., 2005). Subedi et al. (2016) found that water extract of *Acorus calamus* at higher dose (8% W/V) was able to check the growth of the pathogen *Stemphylium botryosum* completely *in vitro*.

Besides the several antifungal activity of the essential oil of palmarosa, lemon grass, and calamus against *S. rolfsii*, it was ineffective in inhibiting the growth of the pathogen in the treated chilli seedling in planta assays. In the initial two days after inoculation, there was no any infection in the treated chilli seedling with essential oil of palmarosa, lemon grass and calamus but after that there was progress of the pathogen growth in the collar region by girdling effect. Later on, in four to five days of inoculation there was complete wilting and collapse of the treated chilli seedling. Chilli seedling in control collapsed within one to two days after inoculation. Essential oils of palmarosa, lemongrass and calamus which performed very well under *in vitro* conditions had 100 percent disease incidence in the chilli seedlings. The ineffectiveness of the essentials

oils in suppressing the disease might be due to the instability of the oil and lesser direct contact of the oil with the pathogen under *in vivo* conditions compared to *in vitro* conditions. The volatile nature of the oil might have reduced their effectiveness under *in vivo* conditions. Not only this, dilution of the essential oils directly with water had caused floating of the oil in the upper layer of the water which also hindered uniform distribution of the oil in the treated chilli seedlings (Amini et al., 2016).

CONCLUSION

Based on the findings, it can be concluded that the essential oils of palmarosa, lemon grass, and calamus exhibited potent antifungal activity against *S. rolfsii* at lower concentration of 500 ppm *in vitro*. However, despite their effectiveness *in vitro*, none of the tested essential oils inhibited pathogen growth *in vivo*. The ineffectiveness of the essentials oils in reducing the disease incidence in chilli seedlings could be due to the instability of the oil and lesser direct contact of the oil with the pathogen under *in vivo* condition compared to *in vitro* condition. The promising antifungal potential of the tested essential oils found effective *in vitro* could be used as an alternative to harmful fungicide with proper formulation development without losing chemical activities by considering their volatile nature in order to manage the disease *in vivo* condition.

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