SCREENING OF ANTIFUNGAL POTENTIAL OF *OCHROBACTRUM CICERI* AGAINST *SCLEROTIUM ROLFSII*

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ABSTRACT

The soil-borne fungus *Sclerotium rolfsii* Sacc., which causes collar rot, is a major constraint of chili (*Capsicum annum* L.) production causing substantial yield loss in Pakistan. Bacterial biocontrol agents are among the favored trends as efficient and eco-friendly agents to mitigate disease stress in modern agriculture. The present study assess *in vitro* antifungal potential of *Ochrobactrum ciceri* against *S. rolfsii* through dual culture, modified dual culture, and novel ring methods. Initially, the pathogenicity of the *S. rolfsii* was confirmed in the chili plants, later antifungal potential of *O. ciceri* was determined. Results of pathogenicity assays revealed that *S. rolfsii* was a virulent pathogen of chili, which produced transparent, thin-walled, and septate hyphae with clamp connections and abundant sclerotia on malt extract agar medium plates. Antifungal assays indicated that *O. ciceri* hold considerable antimycotic activity against *S. rolfsii* by inhibiting 98-100%, 69-70%, and 55-70% of the fungal growth by the novel ring, modified dual culture, and dual culture methods, respectively. The novel ring method was found more effective for rapid assessment of the bacterial antifungal activity as it caused 100% and 98% fungal growth inhibition after 7 and 15 days of incubation, respectively as compared to the conventional dual culture plate assay. It was concluded that for the screening of potential bacterial antagonists against phytopathogenic fungi, the novel ring methods could be utilized as a rapid assessment of bacteria, which not only reduces required resources but is also found as an efficient method for reliable results as compared to conventional dual culture plate assay.

Keywords: Management, Pathogenic fungus, Non-spore forming bacteria

Introduction

A soil-borne phytopathogenic fungus known as *Sclerotium rolfsii* Sacc. (Teleomorph *Athelia rolfsii* (Curzi) C. C. Tu and Kimbr) is a necrotroph and causes Sclerotium wilt, collar or stem rot, foot rot, crown rot, and blight in addition to damping off of seedlings in more than 500 plant species (Nafisa *et al*., 2014; Sana *et al*., 2017; Mahadevakumar and Sridhar, 2021). The fungal survival structures viz., sclerotia may remain dormant in the soil for several years until favorable conditions and easily spread by wind, water, and foot or farm vehicle traffic (Sana *et al*., 2016). Under suitable conditions (25 °C-30 °C and pH 6-7), sclerotia germinate to produce massive hypha, which infects the susceptible

plant (Heller *et al.,* 2013; Sana *et al*., 2017),. During infection, *S. rolfsii* secretes tissue-degrading enzymes e.g. oxalic acid which can incorporate with calcium, separating it from the pectic compounds in plant cell walls, decreasing cell wall pH, and thereby favoring activity of the cell wall-degrading enzymes (Deacon, 2006; Heller *et al.,* 2013).

Maceration interrupts the delivery of water and nutrients in plant tissues, thereby causing wilting, yellowing, and necrosis in the host plant. The plant with collar rot shows a girdle around the collar region, above the ground line. The girdling develops upwards, along with the white mycelium. Wilting takes place within 3-5 days and the complete plant dries up with the abolition of the green canopy. White mycelial growths are visible in infected plant tissues, and they frequently radiate over the soil's surface (Acabal *et al*., 2019).

The chemical fungicides, including mancozeb, carbendazim, hexaconazole, thiram, and carboxin have been used to combat the pathogen (Sekhar *et al*., 2020). However, the indiscriminate use of synthetic agrochemicals is contributing to the deterioration of natural habitats, loss of biodiversity, environmental pollution, and threats to food safety (Shoaib *et al*., 2022). The biological control of fungal diseases through different bacterial strains is less expensive and eco-friendly as compared to chemical fungicides (Awan and Shoaib, 2019; Shoaib *et al*., 2020; Awan *et al*., 2021). Recently, rhizobacteria have received a lot of attention in the modern agricultural system due to their growth-promoting effect and biocontrol potential (Awan and Shoaib, 2019; Awan *et al*., 2021). Rhizobacteria possess distinct mechanisms of action including hyperparasitism, antibiosis, competition, and induced systemic resistance against the phytopathogenic fungi (Awan *et al*., 2021). Many strains of bacteria such as *Rhizobium*, *Ensifer*, *Pseudomonas*, *Bacillus*, *Ochrobacturm*, *Phyllobacterium*, *Herbspirillum*, *Shinella*, *Alcaligenes*, *Azospirillum, Azotobacter*, *Mesorhizobium, Devosia, Clostridium, Enterobacter* and *Klebsiella* have been successfully used to promote plant growth and control diseases in plants throughout the world (Korejo *et al*., 2017). Among them, *Ochrobactrum* (proteobacteria) is present widely in the rhizosphere, and its various species are well-known to elicit and induce systemic resistance in plant systems against various pathogens (Ngoma *et al*., 2014; Priyanka and Nakkeeran, 2019). Therefore, the present study was conducted to check the the antifungal potential of *O. ciceri* (FCBP0727) against *S. rolfsii* through dual culture, modified dual culture, and novel ring methods.

Materials and methods

Pure culture of disease-causing fungus *S. rolfsii* (FCBP1409) was sub cultured on 2% Malt extract agar medium (MEA: 2 g Malt extract and agar in 100 mL of double autoclaved distilled water). The inoculated plates were kept at 28 °C

for 7 days, and the pure cultured plates were preserved at 4 °C for future use. To establish Koch's postulates, *S. rolfsii* was subjected to a pathogenicity test as described by Freeman *et al*. (2002). The sandy-loam soil (sand: 44%, silt: 30%, and clay: 25%) was disinfected with 2% formalin solution, sieved, air-dried, and kept in pots (150 g/pot) (Shoaib *et al*., 2022) followed by transplantation of one-month-old chili seedlings (5 cm in length). The *S. rolfsii* was inoculated in soil by pouring cultural suspension (20 mL) in each of the three holes made around the plant in each pot. The control pots were also prepared similarly but without pathogen application. The data regarding disease incidence was recorded after 15 days of transplantation. Further, the pathogenicity of *S. rolfsii* was verified by re-isolating the same pathogen from the infected chili roots (Shoaib *et al*., 2022).

The antifungal activity of *O. ciceri* against *S. rolfsii* was further assessed in modified dual culture, and novel ring methods (Kumari *et al*., 2021). In dual culture method, *O. ciceri* was streaked at one end of the plate, and placed the mycelial disc at the opposite side. In the modified and novel ring method, a Petri plate with MEA in the middle has 5-mm-diameter *S. rolfsii* mycelial disc inoculated in it, and the plates were streaked with *O. ciceri* 2.5 cm away from the center in a circular pattern and streaked 3 cm away from the center at four sides, respectively. The control plates were inoculated with *S. rolfsii* only. At 28 °C, all Petri plates were incubated in an inverted position and the radial growth of the fungus was measured on the 7th and 15th days of incubation. The degree of antifungal activity was calculated.

$$Degree\ of\ antifungal\ activity\ (\%) = Growth\ in\ control - Growth\ in\ treatment / Growth\ in\ control \times 100$$

The data were analyzed statistically by analysis of variance (LSD) using Statiatix 8.1. Differences were seemed significant at the 0.05% level.

Results

Pathogenicity test of *S. rolfsii*

The infected chili seedlings indicated wilting symptoms along with white cottony mycelia and initially white and later brown sclerotia developed on the root and soil surface (Fig. 1a). The 92% of disease incidence was recorded in the pathogenicity trials of *S. rolfsii*. The infected chili plants became soft with a water-soaked appearance, and the collar region turned black (Fig. 1 b). On synthetic media (MEA), the fungus produced white to pale olive buff colonies, which cover the whole plate very fast (average growth rate of 2.2 cm/day). The abundance of sclerotia was developed and spread throughout the plate within 12 days of incubation. Initially, sclerotia (diameter 0.5 to 1.5 mm, smooth surface) were white, later turned in brown. The hyphae were transparent and thin-walled, while septation occurred with profuse branching and clamp connections. Mycelium was 1.5-3.5 µm in diameter with branches generated

from clamp connections (Fig. 1 c & d), which are the features of *S. rolfsii* .

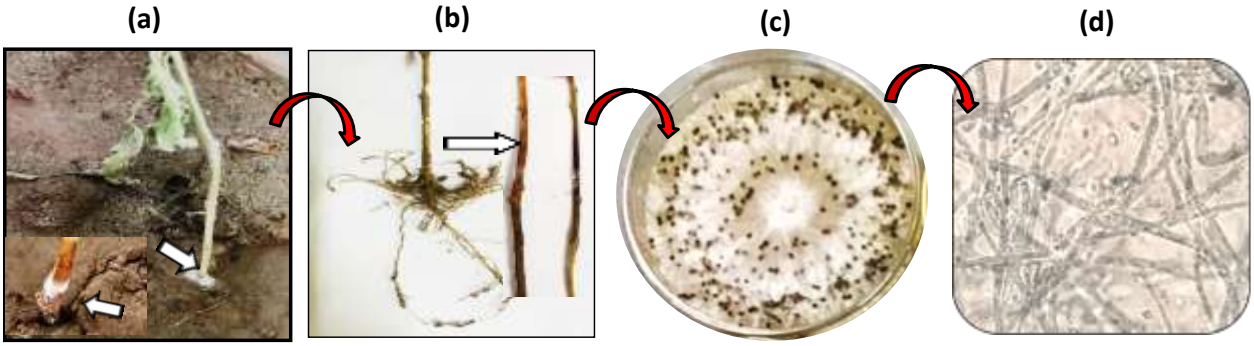

Fig. 1 (a-d): Pathogenicity of *Sclerotium rolfsii* on chili plant. **(a)**: Infected chili plants; (**b)**: Rotting at the collar region; **(c)**: Pure culture of *S. rolfsii*; (**d**): Hyphae with clamp connection at ×40.

Antifungal activity of *O. ciceri* against *S. rolfsii*

The antifungal activity of *O. ciceri* against *S. rolfsii* was further verified by dual culture, modified dual culture, and novel ring methods both at 7 days and 15 days after incubation on 2% MEA at 28 °C. It was observed that on the 7th day of incubation, *O. ciceri* significantly inhibited fungal growth by 100% in the novel ring method followed by 85% and 70% inhibition in modified dual culture, and dual culture methods, respectively as compared to the control (0%). However, on the 15th day of incubation, the antifungal effect of *O. ciceri* was maximum (98%) in the novel ring method followed by modified dual culture (70%) and dual culture methods (55%) fungal growth as compared to the control (Fig. 2; Table 1).

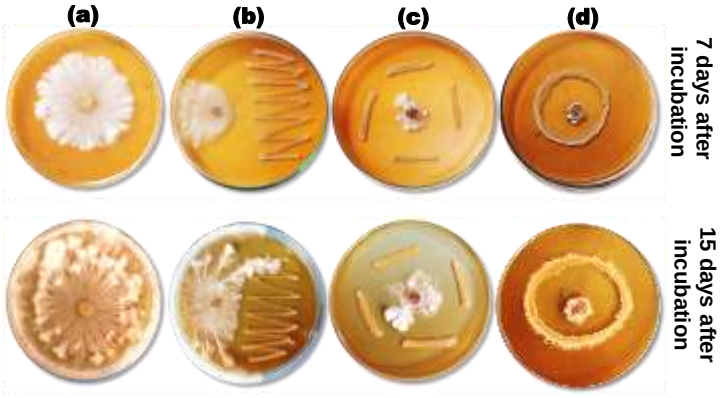

Fig. 2 (a-d): Interaction between *Ochrobactrum ciceri* and *Sclerotium rolfsii* on 2% Malt extract agar medium plates at 7th and 15th days after incubation at 28 °C. **(a)**: *Sclerotium rolfsii* (control); **(b)**: Dual culture method; **(c)**: Modified dual culture method; (**d**): Novel ring method.

Table 1: Antifungal effect of *Ochrobactrum ciceri* on the growth of *Sclerotium rolfsii* by different methods on 2% Malt extract agar plates 7 and 15 days after incubation at 28 °C.

Days after incubation	Fungal growth (cm) and growth inhibition (%) over control		
	Dual culture method	The modified dual culture method	Novel ring method
7	(2.65^{b}) 70%	(1.07^{d}) 85%	(0.0^{f}) 100%
15	(3.16^{a}) 55%	(1.94^{c}) 70%	(0.2^{e}) 97%

Each treatment in the experiment has three independent biological replicates (n = 3). Letters in superscript show a significant difference ($p \leq 0.05$) as determined by the LSD test

Discussion

Massive use of toxic synthetic fertilizers and pesticides in agriculture is harmful to the environment and dangerous to human health. The use of beneficial rhizospheric bacteria is considered an alternative and effective strategic approach, for crop production and plant protection (Shoaib *et al*., 2021). Endophytes facilitate plant growth by protecting plants from plant pathogens and increasing its tolerance

against various biotic and abiotic stresses (Khanna *et al*., 2019).

The present study was based on determining the antifungal activity of *O. ciceri* through the dual culture, novel ring, and modified dual culture methods against *S. rolfsii*, responsible for collar rot disease in *C. annum*. Initially, the pathogenicity of *S. rolfsii* was checked on chili plants, and the results indicated that infection of the pathogen caused browning, yellowing and discoloration of leaf tissue. There were formations of deep dark brown lesions on the stem region of the plant just near the ground. Advanced symptoms developed when the lesion was covered by a radiating white mycelium with rotting underneath it. In later stages of infection, such bodies were produced abundantly on the stem (Sana *et al*., 2016; Sivakumar *et al*., 2016). Nagamma and Nagaraja (2015) also noticed the symptoms of *S. rolfsii* on chickpea plants and observed that the infection starts first at the collar region. Re-isolation of the pathogen from the infected chili plant on MEA produced white to pale olive buff colonies, abundant sclerotia, and microscopically transparent, branched, thin-walled, septate hyphae were observed with the clamp connections Paparu *et al*. (2020) also described similar macro and microscopic features of *S. rolfsii*.

Generally, the dual culture method is taken easy and rapid qualitative assay for first-line screening of bacterial antagonists against fungal pathogens (Shoaib *et al*., 2020, 2021). However, there are other *in vitro* assays like modified dual culture and novel ring methods that can serve as alternatives to the dual culture methods with more authentic results (Kumari *et al*., 2021). Besides, understanding how a bacterial antagonist acts against the *S. rolfsii* by different methods will be helpful in developing crops specific antagonists with multiple disease-suppressive abilities. It was observed that the *O. ciceri* significantly inhibited fungal growth by 100% in the novel ring method followed by 85% and 70% inhibition in modified dual culture, and dual culture methods, respectively as compared to the control 7 days after inoculation. However, there was 98%, 70%, and 55% inhibition in the fungal growth after 15th day of inoculation, in the novel ring, modified dual culture, and dual culture methods, respectively over control treatments. A parallel result was stated by Fakoya *et al*. (2009) where the antifungal activity against *S. rolfsii* was much better in the novel ring method than in the dual culture method. Kumari *et al*. (2021) also reported 100% antifungal activity of *Bacillus* spp. after 7 days of incubation with *S. rolfsii* by a novel ring method, and ascribed it as availability of sufficient space for the bacteria to grow relative to the fungus. In the present study, the higher antifungal potential of *O. ciceri* in novel ring method might ascribed to the direct antagonistic potential of bacteria from all sides, while left with no space/opportunity for the fungus to grow. Furthermore, novel ring methods seem to perform antifungal assay to a

great extent with the rapidness of the results and 100% saving of repetition of the experiments.

In general, *O. ciceri* exhibited a greater antifungal potential of 70-100% against *S. rolfsii* in different methods, which might be associated with the potential of antagonists to solubilize phosphate, produce hydrogen sulfide and also siderophore (Shoaib *et al*., 2020). *O. anthropi* isolated *Ochrobactrum* sp. from the phylloplane of tea and reported antifungal activity against *Exobasidium vexans* under field condition (Sowndhararajan *et al*., 2013), while Chaiharn *et al*. (2009) stated that *O. anthropi* strain isolated from the rice rhizosphere expressed antagonistic activity against *F. oxysporum*. Ngoma *et al*. (2014) indicated the antagonistic activity of *O. intermedium*, *Ochrobactrum* sp. and *O. anthropi* against *F. oxysporum*.

According to Priyanka *et al*. (2018), *O. ciceri* reduced the growth of *Botrytis cinerea* might be due to the production of antifungal compounds (volatile and non-volatile) such as pentadecane and dimethyl trisulfide. Likewise, Shoaib *et al*. (2020) found that *O. ciceri* is the most effective biocontrol agent against *Macrophomina phaseolina* due to their production ability of different antimycotic compounds such as siderophores, phenazines, pyrrolnitrin, hydrogen cyanide, 2, - diacetylphloroglucinol, viscosinamide, pyoluteorin, and tensin.

Conclusions

The present study demonstrated the strong antifungal potential of *O. ciceri* against *S. rolfsii*. The novel ring suppressed the virulent pathogen *S. rolfsii* most effectively compared to dual and modified dual methods. It is proposed that *O. ciceri* can be used as an effective biocontrol agent for the control of diseases caused by *S. rolfsii* in chili.

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