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Optimizing culture conditions for the antagonistic activities of *Trichoderma viride* against *Sclerotium rolfsii* causative agent of southern blight disease of tomato

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ABSTRACT

Aims: *Sclerotium rolfsii* is a pathogen of agricultural crops such as tomatoes. *Trichoderma viride* has been shown to control this pathogen effectively but the optimum conditions of this biocontrol agent need to be determined. This piece of research was therefore aimed at studying the conditions that could be maintained in maximizing the full potential of *T. viride.*

Methodology and results: Malt extract agar was separately supplemented with different carbon sources (glucose, sucrose, maltose, lactose, and mannitol), nitrogen sources (peptone, ammonium nitrate, zinc nitrate and sodium nitrate) as well as salts (NaCl, $CaCl_2$, KI, $BaCl_2$) at different concentrations. Effect of different temperature and pH ranges was also evaluated. All the carbon used supported the growth of both *S. rolfsii* and *T. viride* at all concentrations. The growth of *S. rolfsii* was less than 90 mm at 3% of all the carbon used compared to the plates in which no carbon sources were added whereas the growth of *T. viride* was 90 mm. There were morphological changes in the growth of both *T. viride* and *S. rolfsii* with increase in concentrations of peptone and ammonium nitrate. The greenish colour of *T. viride* was completely lost while *S. rolfsii* became fluffier. For peptone, the percentage reduction ranged from 68.52 to 63.33 while for zinc nitrate and sodium nitrate, the percentage reduction ranged from 85.19 to 80.74 and 55.00 to 43.70 respectively. Also, there was increase in antagonistic property of *T. viride* with the inclusion of salts (NaCl, $CaCl_2$ and KI) at all concentrations used. However, barium chloride was toxic to *T. viride* at 2-5% (w/v). The optimum temperature and pH for the antagonistic property of *T. viride* appeared to be 37 °C and 6.0 respectively.

Conclusion, significance and impact of study: Inclusion of different carbon, nitrogen and salts increased the antagonistic activities of *T. viride* against *S. rolfsii* although lactose appeared to be the best carbon source while zinc nitrate and $CaCl_2$ were the best nitrogen and salt respectively. Barium chloride was toxic to *T. viride* at 2-5% (w/v).

Keywords: Sclerotium rolfsii, *Trichoderma viride*, antagonistic activities, optimum condition

INTRODUCTION

Sclerotium rolfsii Sacc. is a soil borne plant pathogen of a wide range of agricultural and horticultural crops (Darakhshanda *et al*., 2007). *Sclerotium rolfsii* is mainly found in warm regions where it causes root rot, stem rot, wilt and foot rot (Domsch *et al.*, 1980; Farr *et al.*, 1989). This organism is pathogenic to a number of cultivated and non-cultivated plants but rarely on cereals (Sarma, 2002; Maurya *et al.*, 2007) and causes serious yield loss in crops of high economic importance (Maurya *et al*., 2010*)*. The crops that are mostly affected by *S. rofsii* include soybean, peanut, sugarbeet, pepper, tomato and potato while sorghum, wheat, rice, lentil, betelvine, alfalfa, cotton, sugarcane, tobacco, sunhemp, sunflower, chrysanthemum, gladiolus and other ornamental species are less affected (Ansari, 2005). The development of sclerotia is greatly influenced by both living and non living factors such as temperature and pH (Ellil, 1999; Sarma, 2002).

Fungal diseases have been controlled by the application of large quantities of chemical fungicides. However, their extensive use causes serious pollution problem in the environment (Raghunathan and Divakar, 1996). Therefore, there is the need for an alternative (Zegeye *et al*., 2011).

"A number of pythopathogenic fungi such as *Rhizoctonia solani*, *Pythium aphanidermatium*, *Fusarium oxysporum, F. culmorum*, *Gaeumannomyces graminis* var. *tritici*, *Sclerotium rolfsii*, *Phytophthora cactorum, Botrytis cinerea* and *Alternaria* spp. have been effectively controlled by *Trichoderma* species" (Jones and Stewart, 1997; Kucuk and Kivanc, 2003; Dolatabadi *et al*., 2011). *Trichoderma* species are known to secret extracellular

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chitinase, cellulase and β-1,3-glucanase which break which facilitate its penetration into the cytoplasm of the target fungi (Cruz *et al*., 1995).

Other mechanisms of action of *Trichodema* species include production of antifungal agents such as gliotoxin, mycoparasitism, competition for nutrients or space, tolerance to stress, among others (Harman, 2000). Although there are several publications on the antagonistic activities of *T. viride* as well as effect of carbon and nitrogen sources on the mycelial growth and weight of *T. viride*, there is little or no information on the effects of these parameters on its antagonistic properties against *S. rolfsii.* This study was therefore investigated to ascertain positive effect of different carbon, nitrogen and salts sources on the antagonistic potentials of *T. viride* against *S. rolfsii* as well as temperature and pH.

MATERIALS AND METHODS

Trichoderma viride was collected from the Department of Microbiology, Federal University of Technology, Akure (FUTA). This isolate was obtained from yellow maize cob on potato dextrose agar. *Sclerotium rolfsii* was collected from the Department of Crop Soil and Pest Management, FUTA, Akure.

Effect of carbon and nitrogen sources on antagonistic properties of *T. viride*

The dual culture technique was used to determine the ability of *T. viride* to inhibit mycelial growth of *S. rolfsii* as affected by different carbon and nitrogen sources according to the method of Gomathi and Ambikapathy (2011).

Effect of carbon sources

For the carbon source assay, malt extract agar was supplemented with different carbon sources viz: glucose, sucrose, maltose, mannitol and lactose separately at three concentrations viz 1%, 2% and 3% (w/v). Effect of the various carbon sources on the antagonistic activities of *T. viride* was conducted using the previously described method.

Effect of nitrogen sources

Malt extract agar was supplemented on separate Petri dishes with the following nitrogen sources: ammonium sulphate, sodium nitrate, peptone and zinc nitrate at three different concentrations viz 1%, 2% and 3% (w/v). Effect of the various nitrogen sources on the antagonistic activities of *T. viridae* was conducted using the previously described method.

Effect of salt concentration on the antagonistic properties of *T. viride*

Five different concentrations (1, 2, 3, 4, 5% (w/v) of the following salts: sodium chloride, barium chloride, calcium chloride and potassium iodide were used to determine the effect of salt on the antagonistic properties of *T. viride*. Malt extract agar supplemented various concentrations of the salt before sterilization. Effect of salt concentration on the antagonistic activities of *T. viride* was conducted using the dual technique previously described method.

Effect of oxygen on the antagonistic activities of *T. viride*

The dual culture technique was used but incubation was done in an anaerobic jar at 25 °C for 5 days. Two sets of control were done; plates containing *Trichoderma* alone and *S. rolfsii* alone.

Effects of pH and temperature on antagonistic properties of *T. viride*

Effect of pH

Malt dextrose agar with pH levels of 3, 6, 7, 9, 12 were poured into Petri dishes and a 7 mm plug from the margin of actively growing colony of *T. viride* and *S. rolfsii* were placed in opposite direction and were incubated at 25 °C for 5 days (Ghildiyal and Pandey, 2008). Percentage reduction of the mycelial growth of *S. rolfsii* was calculated according to the method previously described.

Effect of temperature

Seven mm plugs from margins of actively growing colonies of *T. viridae* and *S. rolfsii* were placed in opposite direction on sterile malt extract agar. The Petri dishes were then incubated at various temperatures, viz: 4, 25, 37 and 50 °C for 5 days (Ghildiyal and Pandey, 2008). The percentage inhibition was calculated using this formula (Gomathi and Ambikapathy, 2011).

Percentage mycelia growth inhibition of *S. rolfsii*

$$= \frac{C2-C1}{C2} \times 100$$

Where C2: Growth of *S. rolfsii* without *T. viride*
C1: Growth of *S. rolfsii* and *T. viride*

RESULTS

Effect of carbon sources on the antagonistic property of *T. viride*

All the carbon sources (sucrose, maltose, lactose, glucose and mannitol) used supported the growth of the test fungus and antagonist (*T. viride*) at all concentrations. *Sclerotium rolfsii* was able to overgrow *T. viride* of all the carbon sources except lactose at all concentrations. However, it was observed that with increase in incubation period, there was lysis of the mycelium of *S. rolfsii* by *T. viride.* The growth of *S. rolfsii* was less than 90 mm at 3% of all the carbon used compared to the plates in which no carbon sources were added whereas the growth of

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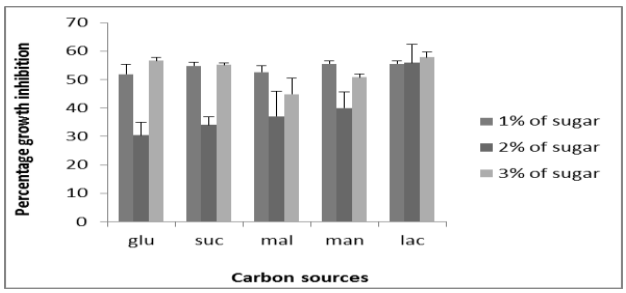

Figure 1: Effect of different carbon sources on the percentage growth inhibition of *S. rolfsii* by *T. viride* on malt extract agar at 25 °C on day 5. Glu, Glucose; Suc, Sucrose; Mal, Maltose; Man Mannitol; Lac, Lactose.

Trichoderma was 90 mm. The percentage reduction of the mycelial growth of *S. rolfsii* is shown in Figure 1.

Effect of nitrogen sources on the antagonistic property of *T. viride*

There were morphological changes in the growth of both *T. viride* and *S. rolfsii* with increase in concentrations of all the nitrogen sources. The greenish colour of *T. viride* was completely lost while *S. rolfsii* became fluffier. The growth of *S. rolfsii* was retarded with increased concentration of nitrogen while *T. viride* was not affected. Percentage growth inhibition of *S. rolfsii* by *T. viride* is shown in Table 1.

Effect of variation in salt concentration on the antagonistic property of *T. viride*

It was observed that there were changes in the morphological characteristics of *T. viride* with increase in salt concentration. However, there was reduction in the growth of *S. rolfsii* from 3 to 5% and the fluffy nature of this fungus. The growth of *S. rolfsii* was greatly reduced at 4% compared to other concentrations on sodium chloride. The growth of *T. viride* was completely inhibited from 2 to 5% of barium chloride (Figure 2). The percentage reduction of *S. rolfsii* by *T. viride* under different concentrations of the selected salts is shown in Figures 3-5.

Effect of oxygen on the antagonistic property of *T. viride*

Trichoderma viride was also antagonistic to *S. rolfsii* when incubated anaerobically at 25 °C with percentage inhibition of 58.89. However, there was inhibition of conidial formation of *Trichoderma viride* but *S. rolfsii* grew profusely even in this condition. It was also observed that when *T. viride* was incubated aerobically afterwards, there was formation of conidia.

Table 1: Comparative influence of different nitrogen sources on the antagonistic properties of *T. viride* against *S. rolfsii*.

Nitrogen sources	Mycelial growth and percentage reduction of *S. rolfsii* with increase in concentration of nitrogen sources					
	Mycelial growth (mm)			Percentage reduction of *S. rolfsii*		
	1% (w/v)	2% (w/v)	3% (w/v)	1% (w/v)	2% (w/v)	3% (w/v)
Sodium nitrate	28.33±0.58	27.33±2.89	25.33±4.98	55.00±1.85^{c}	52.87±0.64^{c}	43.70±1.28^{c}
Zinc nitrate	13.33±1.53	16.33±3.21	17.33±4.93	85.19±1.70^{a}	81.85±3.57^{a}	80.74±5.49^{a}
Peptone	28.33±1.16	30.67±0.58	33.00±3.61	68.52±1.28^{b}	65.93±0.64^{b}	63.33±4.01^{b}
Ammonium nitrate	15.33±2.30	14.33±0.58	28.33±2.08	82.96±2.57^{a}	84.07±0.64^{a}	68.52±2.31^{b}

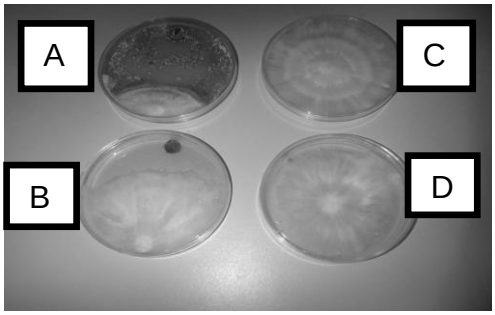

Figure 2: Effect of barium chloride on the antagonistic property of *T. viride* against *S. rolfsii.* A, Growth of *T. viride* and *S. rolfsii* at 1% of barium chloride; B, Growth of *T. viride* and *S. rolfsii* at 2% of barium chloride; C, Growth of *S. rolfsii* at 1% of barium chloride; D, Growth of *S. rolfsii* at 2% of barium chloride.

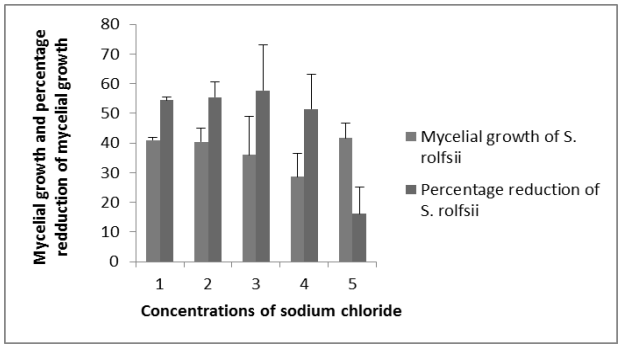

Figure 3: Effect of sodium chloride on the antagonistic property of *T. viride* against *S. rolfsii* at 25 °C on malt extract agar. 1, 1% sodium chloride; 2, 2% sodium chloride; 3, 3% sodium chloride; 4, 4% sodium chloride; 5, 5% sodium chloride.

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Effect of temperature on the antagonistic property of *T. viride*

The growth rate of *S. rolfsii* was greatly reduced at 4 °C and 37 °C. However, *T. viride* grew well at 37 °C but there was inhibition in its conidiation and growth rate at 4 °C. Neither *T. viride* nor *S. rolfsii* grew at 50 °C. There were significant differences in the antagonistic property of *T. viride* (Table 2).

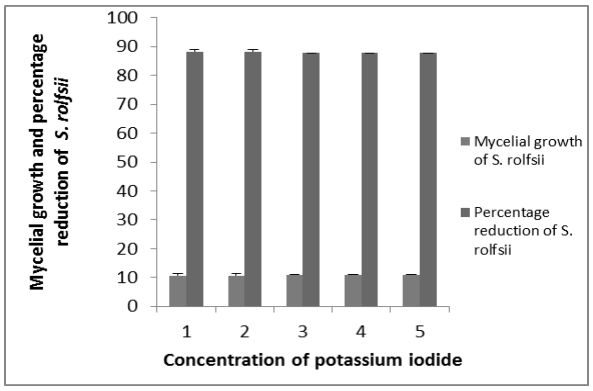

Figure 4: Effect of potassium iodide on the antagonistic property of *T. viride* against *S. rolfsii* at 25 °C on malt extract agar. 1, 1% potassium iodide; 2, 2% potassium iodide; 3, 3% potassium iodide; 4, 4% potassium iodide; 5, 5% potassium iodide.

Effect of pH on the antagonistic property of *T. viride*

Sclerotium rolfsii was able to grow at pH 3, 6, 7, 9 but the growth was hampered at pH 12. The antagonistic property of *T. viride* was only observed at pH 3, 6 and 7. Neither *T. viride* nor *S. rolfsii* was able to overgrow each other at pH 12 (Table 3).

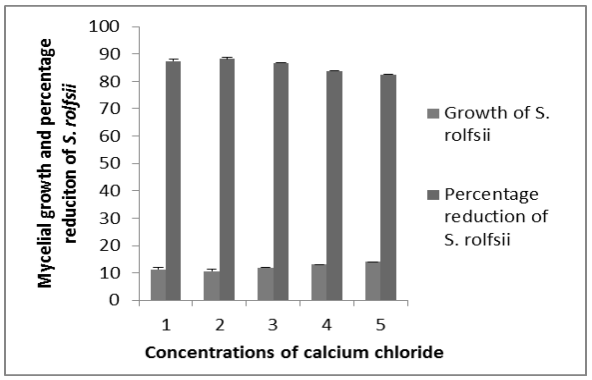

Figure 5: Effect of calcium chloride on the antagonistic property of *T. viride* against *S. rolfsii* at 25 °C on malt extract agar. 1, 1% calcium chloride; 2, 2% calcium chloride; 3, 3% calcium chloride; 4, 4% calcium chloride; 5, 5% calcium chloride.

Table 2: Effect of temperature on the antagonistic property of *T. viride* against *S. rolfsii* on MEA.

Temperature (°C)	Mycelial growth of *Trichoderma viride* (mm)	Mycelial growth of *Sclerotium rolfsii* (mm)	Percentage reduction of *S. rolfsii* over control
4	25.00±0.00	10.00±0.00	0.00
25	43.33±2.89	35.00±4.58	41.67±7.67
37	48.67±3.22	41.33±3.22	36.41±4.94
50	0.00	0.00	0.00

Table 3: Effect of pH on the antagonistic property of *T. viride* against *S. rolfsii*.

pH range	Mycelial reduction of *T. viride*	Mycelial reduction of *S. rolfsii*	Percentage inhibition of *S. rolfsii* over control
3	54.00±3.46^{a}	36.00±3.46^{b}	20.00±7.70^{c}
6	59.33±1.16^{a}	30.67±1.16^{c}	40.37±6.29^{a}
7	56.00±1.00^{a}	34.00±1.00^{b}	29.62±2.46^{b}
9	35.00±t5.00^{b}	49.33±1.16^{a}	-2.13±3.69^{d}
12	15.33±0.58^{c}	25.33±0.58^{d}	15.56±1.93^{c}

DISCUSSION

Trichoderma viride grew well on all the carbon sources. Application of additional carbon source did enhance the antifungal activity. Similar observation has been made by Sesan and Oancea (2005). Chovanec *et al*. (2001) also observed conidiation in *T. viride* cultures grown on 30 out of 32 carbon sources. Antagonists' mode of action might be a combination among competitive exclusion, preemptive colonization and the production of anti-fungal substances (Nalisha *et al*., 2006).

Trichoderma viride was tolerant to higher concentration of NaCl, $CaCl_2$ and KI although there was colour change compared to the medium unsupplemented with salts. Most *Trichoderma* strains used as biofungicides have low osmotolerance potentials (Mohamed and Haggag, 2006). Environmental factors associated with climate change, especially temperature and soil salinity, may influence plant pathogens,

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biocontrol agents, and mechanisms of their interactions (Gal-Hemed *et al*., 2011). High salinity may increase the severity of diseases caused by a variety of plant pathogens (Hasegawa *et al*., 2000; Triky-Dotan *et al*., 2005), and the search for new *Trichoderma* strains capable of overcoming extreme environmental conditions is timely. Mohamed and Haggag (2006) reported that *T. harzianum* is sensitive to high concentration of salt contrary to the present work. Gal-Hemed *et al*. (2011) observed that *Trichoderma atroviride* and *T. asperelloides* exhibited a very good growth adaptation to saline environments *in vitro* and they can therefore be used as biological control agents of plant growth in saline soils. Ghildiyal and Pandey (2008) also observed that *T. harzianum*, *T. koningii* and *T. viride* isolated from soil samples of glacier sites in high altitudes of Indian Himalaya could tolerate salt concentration up to 5% (w/v). Fungi have been shown to develop extrusion systems to keep levels of intracellular sodium below concentrations toxic to the cell (Gunde-Cimerman *et al*., 2009). Furthermore, adaptation to higher salt concentrations can be used to reduce the probability of fungal/bacterial contamination in *Trichoderma* stocks intended for liquid formulations in the on-site cultivation of strains (Harman *et al*., 2010). However, $BaCl_2$ was toxic to *T. viride* from concentration 2-5%. Knowledge of the prevalence of environmental conditions, both climatic and edaphic, in the habitat of a given organism may be useful for exploitation of the potential applications associated with the organism (Ghildiyal and Pandey, 2008). Certain strains of *Trichoderma* sp. including *T. viride* are known to be restricted to the areas identified by low temperature. There was reduction in growth of both *T. viride* and *S. rolfsii* at 4 °C though the growth of *T. viride* was higher.

The growth of *T. viride* was least at pH 12. Sesan and Oancea (2005) observed that *T. hiazianum* showed the poorest growth at pH 13 while the best growth was at 4.0-5.5. However, Ghildiyal and Pandey (2008) observed optimum pH of *Trichoderma* species to be 5.5. The optimum pH in this research was 6.0.

CONCLUSION

There was increase in the antagonistic activities of *T. viride* supplemented with various carbon and nitrogen sources. *Trichoderma* viride was halotolerant to NaCl, KI and $CaCl_2$ but halosensitive to $BaCl_2$ from 2-5%. The optimum temperature and pH for antagonistic activities of *T. viride* was 37 °C and pH 6.

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