World Journal of Microbiology and Biotechnology 7, 494–501

Effects of temperature, pH and water potential on growth of four fungi with disease biocontrol potential

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Effects of temperature, pH and water potential on biomass production or hyphal extension of *Gliocladium virens* (G20) and three *Trichoderma* isolates were determined *in vitro*. Optimum biomass production occurred between 20 and 30°C and at pH ranges between 4.6 and 6.8. Two isolates of *T. viride* grew at 5°C and *G. virens* grew at 35°C but no isolates grew at 40°C. Hyphal extension rates and conidial germination of all fungi declined with decreasing water potential over the range −0.7 to −14.0 MPa. In general, growth rates for each isolate were lower on potato/dextrose agar with water potential adjusted with polyethylene glycol than when adjusted with NaCl or glycerol. No mycelial growth or spore germination occurred on agar at −14.0 MPa.

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Allium white rot (AWR) disease of onion and related crop species caused by *Sclerotium cepivorum* Berk. results in severe crop losses in many parts of the world (Utkhede & Rahe 1980). The problem is increasing owing to the lack of reliable methods of cultural control and the loss of dicarboximide fungicide effectiveness (Entwistle 1986; Walker *et al.* 1986). There is thus considerable potential for biological control of this disease.

Very few biological control agents are commercially available for the control of plant pathogens (Ayers & Adams 1981; Chet *et al.* 1984; Kerr & Tate 1984; Whipps 1986; Lynch 1988). In part this is due to erratic or incomplete levels of control. For example, a number of *Bacillus* isolates reduced AWR in initial field trials but failed to reduce disease in subsequent years (Utkhede & Rahe 1983; Rahe 1986). Consequently, biological control has often been considered to be less effective and a greater risk than chemical control. This could perhaps be improved if physiological and ecological attributes of potential biocontrol organisms were determined before field use. This would enable predictions of their ability to grow and survive in soil to be made and assist in improving inoculum production.

A series of *in vitro* screens have detected four fungi with potential biological control activity against AWR (Jackson *et al.* 1991a). Methods of inoculum production and some physiological growth parameters have been examined already (Jackson *et al.* 1991 b,c,d) and this paper extends these studies to the effects of temperature, pH and water potential on growth and spore germination.

Materials and Methods

Fungal Isolates

The antagonists used in this study were: *Gliocladium virens* Miller, Giddens & Foster (G20) which was provided by R.D. Lumsden, Beltsville, USA; *Trichoderma viride* Pers.ex Fr. (IMI 322659) which was isolated from a sclerotium of *Sclerotinia sclerotiorum* Lib. de Bary; *T. viride* Pers. ex Fr. (IMI 322663) and *T. pseudokoningii* Rifai (IMI 322662) which were isolated from *Allium* white-rot-diseased onion bulbs.

All antagonists were maintained on potato/dextrose agar (PDA; Oxoid CM

139) and routinely sub-cultured to maintain a supply of actively growing and sporulating cultures which were used as inoculum sources for studies on growth requirements.

Requirements for Growth

Temperature. Linear hyphal extension rates (mm/day) of the four fungal isolates were determined between 5 and 40°C on PDA (10 ml) in 9 cm-diameter Petri plates. For each treatment, three replicate plates were inoculated with agar discs (5 mm-diameter) taken from the edge of actively growing cultures with a sterile cork-borer.

pH. The effects of pH on biomass production of the four fungal isolates in molasses-yeast medium were determined in static liquid culture using a molasses-yeast medium already known to support mycelial growth of the four fungal isolates (Jackson *et al.* 1991c). Appropriate amounts of citrate phosphate buffer (Gomori 1955) were added to the medium to give molasses (United Molasses Ltd, Dagenham, UK) at 3% (w/v) and pressed yeast [The Distillers (Yeast) Co., Surrey, UK] at 0.5% (w/v) with pH ranges between 2.5 and 7.4. The original pH of unbuffered molasses yeast medium was 5.5. Medium was sterilized by autoclaving at 120°C and 103.4 kPa for 15 min. Three replicate Erlenmeyer flasks (250 ml) containing 45 ml of each test medium were inoculated with 1 ml sterile distilled water containing 1×10^5 conidia taken from fungal colonies grown on PDA. After 7 days incubation at 25°C, the mycelial mats were harvested by vacuum filtration on to pre-weighed filter papers (Whatman No. 4) and oven-dried for 48 h at 80°C.

Water potential. The water potential of PDA was adjusted osmotically with the non-electrolyte glycerol or the electrolyte sodium chloride (NaCl). The matric potential of PDA was adjusted with polyethylene glycol 6000 (PEG). In addition, distilled water agar (DWA) [20 g Technical agar No. 3 (Oxoid L13) in 1 litre distilled water] was osmotically adjusted by the addition of glycerol only. Quantities of either glycerol, NaCl or PEG were added to media to give required water potentials in the range of −0.7 to −14.0 MPa (Lang 1967; Michel & Kaufmann 1973; Dallyn & Fox 1980; Magan *et al.* 1989).

For all experiments, 10 ml of medium was added to sterile plastic Petri dishes using a Jencons Dispenser. Solidified agar media that were adjusted with either

Table 1. Effects of temperature on linear extension rates (mm/day) of *G. virens* and *Trichoderma* spp. on PDA.

Antagonist	Temperature (°C)						
	5	10	15	20	25	30	35
G. virens (G20)	0.0*	3.1	4.5	8.1	14.1	14.0	5.4
T. pseudokoningii (IMI 322662)	0.0	5.3	8.5	16.3	25.2	24.7	0.0
T. viride (IMI 322659)	0.3	12.0	13.3	18.3	15.7	2.4	0.0
T. viride (IMI 322663)	0.5	8.5	12.6	16.5	17.7	2.0	0.0
SED (62 df)†	0.35						

* Values are means of 3 replicates.

† SED is the standard error of the difference between two means derived by analysis of variance; in particular, the difference between two means divided by its SED gives the appropriate *t*-value (a sig. difference at the $p = 0.05$ level is given by approximately 2 × SED). df = degrees of freedom in analysis of variance.

(There was no growth of any isolate at 40°C.)

glycerol or NaCl were inoculated in the same way as for the temperature studies. However, a cellophane disc (PT600, 9 cm diameter) was placed on to the surface of medium which was adjusted matrically with PEG before inoculation because this medium did not solidify. Three replicate plates of each treatment were placed in polyethylene bags to prevent water loss and incubated at 25°C. During the incubation period hyphal extension was measured and linear extension rates (mm/day) determined.

In spore-germination studies, 10 day-old conidia were removed from PDA-grown colonies by adding 10 ml sterile water to Petri dishes and rubbing with a glass rod. Samples (0.5 ml) were spread over the surface of DWA and PDA adjusted with glycerol to give 50 to 100 spores in a microscope field where the ×10 objective was used. The percentage germination of conidia was estimated on three replicate plates after 18, 40, 68 and 118 h at 25°C (Brian & Hemming 1945). Conidia were considered to have germinated when the germ tube length was equal to or greater than the spore width.

Results

Temperature

Temperature significantly affected the linear hyphal extension rates of all four fungal isolates (Table 1). *G. virens* (G20) grew over 10 to 35°C with a maximum extension rate of 14 mm/day at both 25 and 30°C. No growth occurred at 5 and 40°C. *T. pseudokoningii* (IMI 322662) grew from 10 to 30°C with a maximum extension rate of 25 mm/day at both 25°C and 30°C. Both isolates of *T. viride* (IMI 322659 & IMI 322663) grew between 5 and 30°C, although the optimum for *T. viride* (IMI 322659) was 20°C compared with 25°C for *T. viride* (IMI 322663). Both had optimum extension rates of 18 mm/day.

pH

Mean dry weights of fungal biomass produced in unbuffered control flasks were 333, 266, 348 and 365 mg for *G. virens* (G20), *T. pseudokoningii* (IMI 322662), *T. viride* (IMI 322659) and *T. viride* (IMI 322663), respectively (Table 2). Maximum biomass production by all fungi occurred at pH values between 4.6 and 6.8 with lowest biomass production taking place at the extremes of pH tested (2.5 and 7.4). *T. viride* (IMI 322659) showed a tendency for better growth at more neutral pHs whereas *T. viride* (IMI 322663) grew better at more acid pHs.

Table 2. Effects of pH on biomass production per flask by *G. virens* (G20) and *Trichoderma* spp. after 7 days incubation in molasses-yeast medium at 25°C.

Antagonist	pH							
	5.5*	2.5	3.2	3.8	4.6	5.6	6.8	7.4
	Cell dry wt (mg/45 ml)							
G. virens (G20)	333†	214	294	352	393	329	329	203
T. pseudokoningii (IMI 322662)	266	236	260	275	306	313	292	190
T. viride (IMI 322659)	348	284	293	297	358	428	403	368
T. viride (IMI 322663)	365	305	363	358	389	376	296	260
SED (62 df)‡	15.04							

* Control with no buffer added.
† Values are means of 3 repicates.
‡ See footnote to Table 1 for definitions.

Water Potential

The hyphal extension rates of all fungi declined with decreasing water potential over the range −0.7 to −14.0 MPa (Table 3). In some instances, extension rates were higher at −0.7 MPa compared with that on control PDA (−0.3 MPa) or DWA (−0.1 MPa) plates. No fungus grew on PDA or DWA adjusted to a water potential of −14.0 MPa with glycerol. *G. virens* (G20) grew on PDA and DWA at a water potential of −7.0 MPa adjusted with glycerol but not on PDA adjusted with NaCl or PEG. In contrast, the *Trichoderma* spp failed to grow only at −7.0 MPa when the water potential was adjusted with PEG. On DWA adjusted with glycerol, the densities of colonies produced was very sparse in comparison with the colonies produced on PDA adjusted with glycerol.

Conidial germination was slower on DWA adjusted with glycerol than on PDA adjusted with glycerol (Figure 1). After 118 hours' incubation on PDA, complete spore germination had occurred at all water potentials tested down to −7.0 MPa for all isolates. No germination occurred at a water potential of −14.0 MPa. On DWA, decreased water potential had a more severe effect on spore germination after 118 hours. *G. virens* (G20) was the only isolate which gave complete conidial germination at −4.2 MPa. At this water potential spore germination of the

Table 3. Effect of water potential on radial extension rate (mm/day) of *G.virens* (G20) and *Trichoderma* spp. on agar at 25°C.

Medium	Antagonist	Water potential (−MPa)						
		PDA/ DWA	0.7	1.4	2.8	4.2	7.0	14.0
PDA + PEG	*G. virens* (G20)	15.7*	13.2	6.4	1.9	0.0	0.0	ND
	T. pseudokoningii (IMI 322662)	23.3	19.8	12.9	5.5	2.2	0.0	ND
	T. viride (IMI 322659)	15.2	15.5	10.5	3.4	1.2	0.0	ND
	T. viride (IMI 322663)	15.3	14.8	12.2	4.2	1.7	0.0	ND
	SED (46 df)†	0.52						
PDA + NaCl	*G. virens* (G20)	16.0	17.6	10.9	5.4	3.2	0.0	ND
	T. pseudokoningii (IMI 322662)	24.7	22.0	17.7	12.9	6.3	0.4	ND
	T. viride (IMI 322659)	15.0	15.0	10.7	7.7	5.4	1.0	ND
	T. viride (IMI 322663)	17.0	17.4	13.8	9.9	6.5	1.6	ND
	SED (46 df)	0.31						
PDA + glycerol	*G. virens* (G20)	13.9	10.0	5.8	4.0	2.8	1.2	0.0
	T. pseudokoningii (IMI 322662)	24.5	20.0	16.5	11.8	7.5	1.0	0.0
	T. viride (IMI 322659)	16.7	16.3	11.2	9.2	8.0	3.0	0.0
	T. viride (IMI 322663)	15.7	16.3	13.0	11.8	7.8	2.2	0.0
	SED (54 df)	0.34						
DWA + glycerol	*G. virens* (G20)	18.2	15.7	12.3	6.5	4.0	0.5	0.0
	T. pseudokoningii (IMI 322662)	12.3	9.2	8.0	4.8	3.0	1.0	0.0
	T. viride (IMI 322659)	8.0	8.8	8.0	5.0	4.0	1.0	0.0
	T. viride (IMI 322663)	10.5	8.5	6.5	5.0	3.4	1.8	0.0
	SED (54 df)	0.21						

* Values are means of 3 replicates.
† See footnote to Table 1 for definitions.
ND: not determined.

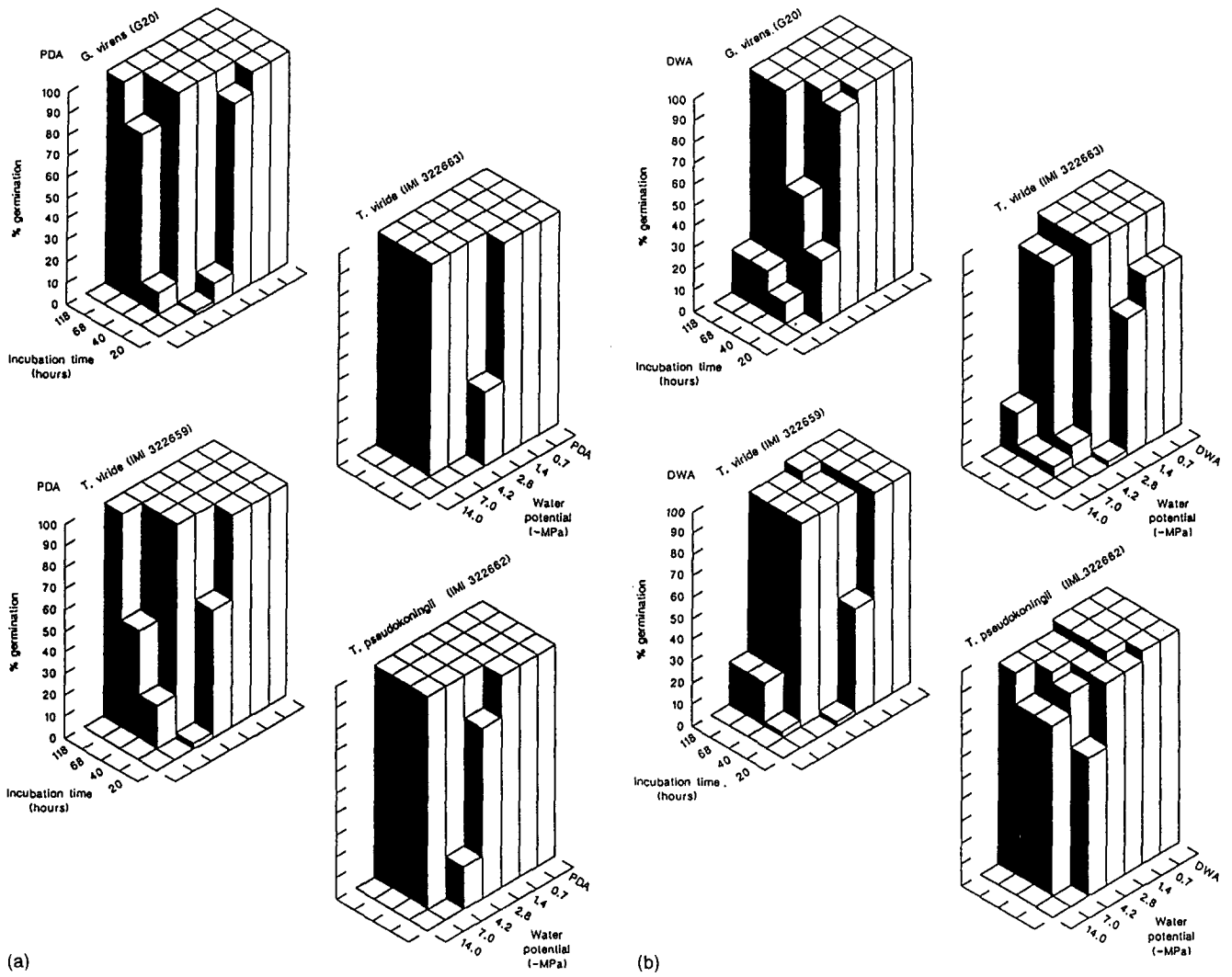

Figure 1. Effect of water potential on conidial germination on (a) potato/dextrose agar and (b) distilled water agar at 25°C.

Trichoderma spp. ranged from 90 to 95%. No germination occurred on DWA plus glycerol at −14.0 MPa.

Discussion

This investigation has identified the temperature, pH and water potential optima for growth of four potentially useful biological control agents. The isolates are typical mesophiles with optimum linear extension between 20 and 35°C. Similar temperature optima have been found with other isolates of these species of fungi (Ward & Henry 1961; Danielson & Davey 1973; Komatsu 1976). The *T. viride* isolates grew at 5°C and may be useful for biological control at low temperatures which are not generally associated with the use of *Trichoderma* and *Gliocladium* species (Papavizas 1985). Tolerance to low temperatures has been observed in some other isolates of *T. viride* and *T. pseudokoningii* (Danielson & Davey 1973; Tronsmo & Dennis 1978) and one of these isolates, applied as a post-harvest dip, has been used to control storage rots of carrots for up to 9 months at 0°C (Tronsmo & Hoftun 1984; Tronsmo 1985). Importantly, all four isolates in this study grew between 10 and 20°C, the range over which *S. cepivorum* is most active in the field (Walker 1926; Adams & Papavizas 1971), and consequently have the potential for active growth at a time when control is required.

The four antagonists produced less biomass below pH 4 and above pH 7 than between pH 4.6 and 6.8. Danielson & Davey (1973) also found that biomass production of *T. pseudokoningii* (T-49) was adversely affected by either extreme acidity or conditions approaching neutrality. Previously, growth of *T. viride* has been shown to occur in the pH range 1.5 to 9.0, the optimum being in the range pH 4.5 to 5.5 (Ward & Henry 1961; Brown & Halstead 1975; Gutter 1963; Komatsu 1976). No information relating to pH effects on the growth of *G. virens* has previously been reported. The pH of soils infected with *S. cepivorum* generally range from 5 to 7 (Tims 1943; 1947), although crop infection has occurred in soil at pH 8 (Adams & Papavizas 1971). Consequently, growth of these antagonists is unlikely to be affected by pH in soils used to grow onions.

Hyphal extension rates declined with decreasing water potential with the minimum for growth lying between −7 and −14 MPa for each isolate. Similar results have been obtained for a range of soil fungi including *Trichoderma* spp (Magan & Whipps 1988; Whipps & Magan 1987). In this study at 25°C, optimal growth occurred at water potential between −0.1 and −0.7 MPa on both PDA and DWA. At 10°C and 20°C on straw agar, Magan & Lynch (1986) also found that most of the fungal species examined, including *T. viride* and *G. virens*, grew optimally at these water potentials. Further, the four isolates in this study were also more tolerant of osmotic than matric potential, with no growth occurring at −7 MPa with media adjusted with PEG, confirming observations of several plant pathogenic fungi (Adebayo & Harris 1971; Brownell & Schneider 1985). Magan (1988) observed a similar effect on the germination of spores and germ tube extension of *Fusarium culmorum, Trichoderma* and *Penicillium* spp.

Medium adjusted to low water potential (−14 MPa) with glycerol was inhibitory to conidial germination. A number of workers have obtained similar inhibition of conidial germination on media with a high salt content (Borut & Johnson 1962; Danielson & Davey 1973; Gindrat 1976). Infection of onion seedlings by *S. cepivorum* is confined to soil matric potentials between −0.0045 MPa and −0.0003 MPa whereas the germination of sclerotia in soil is optimal at −0.03 MPa but will occur at water potentials down to −1.25 MPa (Crowe & Hall 1980). The data produced in this investigation demonstrate that *G. virens* (G20) and the species of *Trichoderma* can grow under moisture conditions which allow *Allium* infection and sclerotium germination of the white rot pathogen.

Conditions favouring the growth of the antagonists and infection of *Allium* by *S. cepivorum* are similar. This is important as, for disease development to be suppressed, it is necessary for the antagonist to be active at the same time as the pathogen. Therefore, the antagonists studied have some useful attributes which suggest their suitability for application to the natural environment for the biological control of *Allium* white rot.

Acknowledgements

We thank Drs A.R. Entwistle and R.T. Burchill (IHR-Wellesbourne) for their co-operation and the Agricultural Genetics Company for financial support.

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(Received 4 February 1991; accepted 16 February 1991)