RAPID EXTRACELLULAR ENZYME ASSAYS FOR SCREENING POTENTIAL ANTISAPSTAIN BIOLOGICAL CONTROL AGENTS

Brenda J. McAfee
Canadian Forest Service
Natural Resources Canada
Ottawa, Ontario K1A 0E4

Benjamin E. Dawson-Andoh†
Division of Forestry
West Virginia University
Morgantown, WV 26506-6125

Maria Chan
Canadian Food Inspection Agency
Health Canada
Nepean, Ontario K2H 8P9

Roger Sutcliffe
Commercial Chemicals Evaluation Branch
Environment Canada
Hull, Quebec K1A 0H3

and

Rawle Lovell
Forintek Canada Corp.
Ste-Foy, Quebec G1P 4R4

(Received September 2000)

ABSTRACT

A Rapid Agar Plate Screening Assay (RAPSA) was developed and optimized for assaying individual extracellular enzymes produced by potential biological control agents and sapstain fungi. The RAPSA, which uses culture filtrates rather than agar plugs inoculated with actively growing fungi as used in the classical screening method, was more sensitive in detecting activity, for all extracellular enzymes screened, with the exception of chitinase, for the majority of the fungi tested. The assay was used to screen potential biological control fungi based on comparison of extracellular enzyme profiles of ten potential antisapstain biological control fungi and three sapstain fungi, grown in liquid cultures containing either glucose, hemlock sawdust, or cell wall of the sapstain fungus *Ophiostoma piceae* as a carbon source. Based on extracellular enzymes profiles, biological control fungi and sapstain fungi were classified into three groups. Group I fungi produced the greatest enzyme activity when glucose was included in the medium. Group II fungi produced equally good activity with sawdust and glucose, while Group III produced high activity with both sawdust and cell wall while enzyme activity with glucose was not consistent.

Five biological control candidates, *Gliocladium viride* 623E, *G. roseum* 784A, *G. virens* 258C, *G. roseum* 321M, *G. virens* 258D, in descending order, demonstrated the full spectrum of extracellular enzyme activity screened, irrespective of the growth medium. Production of extracellular enzymes in a minimal medium augmented with sawdust or cell wall is an indicator of secondary resource capability. *Gliocladium viride* 623E and *G. virens* 258C demonstrated high extracellular enzyme production in both of these media. On this basis, these two fungi were judged to show the greatest potential as biological control agents. *Mariannea elegans* 386E and *G. solani* 810A showed the least potential.

† Member of SWST.

Wood and Fiber Science, 33(4), 2001, pp. 648–661

Keywords: Bioprotection, sapstain and biological control fungi, extracellular enzymes, rapid agar plate assay for enzyme screening.

INTRODUCTION

Sapwood of freshly sawn lumber is a nutritional bonanza for many Ascomycetes and Fungi Imperfecti (Dawson-Andoh and Morrell 1997). Many of these fungi cause sapstain and mold discoloration of green lumber with considerable loss in revenue to the producer. Sapstain and mold discoloration of green lumber are currently controlled through the application of biocides. However, increasing concern about the potential negative impact of biocides has led to the search for alternative methods of control. A promising alternative is biological control (Dawson-Andoh and Morrell 1997; McAfee and Gignac 1997; Yang and Rossignol, 1999).

Biological control agents, unlike biocides, are living organisms that must grow, colonize the wood substrate, and exclude undesirable microflora if they are to be effective when applied. Efficient biological control agents must therefore exhibit excellent primary and/or secondary resource capture. Primary resource capture is a process of gaining access to, and influence over, an available resource, e.g. wood substrate (Rayner and Todd 1979). This process is dependent on the capacity of the fungi to produce enzymes favoring resource capture, good spore germination, and rapid mycelial growth rates. Secondary resource capture is a measure of an organism's ability to invade and capture portions of wood substrate already colonized by competing microflora via mycoparisitism or production of antibiotics.

Understanding the mechanisms underlying a biological control process is essential to the development of a strategy for the process of strain improvement, formulation, and delivery of the bioprotection or biological control agents (Papavizas 1985; Fravel 1988; Lumsden and Lewis 1989; Lewis et al. 1989; Lynch 1990; Lewis and Papavizas 1991). It is essential that biological control agents designed for use on wood do not break down the lignocellulosic complex that makes the major component of the wood cell wall. However, these organisms must be capable of utilizing simple carbohydrates and nonrefractory nutrients in wood as part of the primary resource capture process. Thus, primary resource capture by biological control fungal agents requires the production of extracellular enzymes (ECE) such as pectinases, amylases, cellulases, and cellobiases, and hemicelluases including xylanases, galactomannanases, and glucomannanases. Extracellular enzymes involved in secondary resource capture include fungal cell-wall lyctic enzymes such as α- and β-glucanases, chitinases, and also mannanases (Aronson 1965; Villanueva 1966). Proteases and lipases may also be involved in both primary and secondary resource capture.

When fungi are grown on solid agar medium, extracellular enzymes are secreted into the agar around the fungal colony. If an appropriate substrate is incorporated into the medium, presence of the enzyme activity can be detected by observing the degradation of the substrate around the fungal mycelia. This approach is employed in several classical extracellular enzyme screening procedures (Hankin and Anagnostakis 1975; Hagerman et al. 1985). Similar approaches incorporate substrates coupled with dyes such as remazol brilliant blue and azure. However, all such methods suffer from two major limitations: the dense growth of fungi makes observation of enzyme production difficult, and the production and activity of enzymes are pH-dependent. The method described in this study modifies the Wood and Weisz (1987) procedures to overcome these two limitations by growing the fungus in a defined liquid media adjusted to specific pH levels and by evaluating enzymatic activity using filtered growth media.

The rapid agar plate screening assay (RAPSA), described here, compares extracellular activity of sapstain and potential biological control fungi by applying filtered liquid

growth media onto filter paper discs placed on solid agar media containing target enzyme substrates. Preliminary testing of temperature, effect of substrate concentration in the solid media, incubation time, buffer types, and pH of solid agar was carried out to determine optimum assay conditions for each enzyme. The assay was then used to compare extracellular enzyme profiles produced by potential biological control and sapstain fungi when grown in the presence of glucose, sawdust, or cell walls of a sapstain fungus.

MATERIALS AND METHODS

Growth and maintenance of test fungi

Ten potential fungal biological control isolates from Forintek Canada Corporation (Ste-Foy, Quebec), *Mariannaea elegans* (Corda.) Arnaud ex Samsom 386E, *Trichoderma pseudokoningii* Rifai 228B, *Trichoderma viride* Rifai 161I, *Gliocladium roseum* Bainier 321A, 321M, 321U, *G. solani* (Harting) Petch, 810A, *Gliocladium viride* Matr. 623, *Gliocladium virens* (Miller), Giddens & Foster 258C, 258D and three sapstain fungi: *Alternatria alternata* (Fr.) Keissler 2H, *Aureobasidium pullulans* (d By.) Arnaud 132Q, *Ophiostoma piceae* (Muench) H. & Syd. 387I were used in this study. A known cellulase producer, *Trichoderma harzianum* Rifai 160M, was included as control.

Sensitivity of RAPSA compared with classical enzyme detection tests

The sensitivity for the detection of extracellular enzymes was compared when test fungi were grown directly on an agar growth medium containing an enzyme substrate (classical method) with those grown in a liquid medium where the culture filtrate was transferred to a medium containing the enzyme substrate (RAPSA).

Extracellular enzymes were assayed by a modification of the classical agar diffusion assays (Hankin and Anagnostakis 1975; Kjoller and Struwe 1980; Donly and Day 1984), where the fungi are inoculated on solid medium with enzyme target substrates made up in a basal medium. Fungi from maintenance slants were inoculated onto malt agar (MA) plates (oatmeal agar plates for *O. piceae*) and incubated at 25°C. After 2–3 weeks, mycelial plugs (8 mm in diameter) were cut sub-marginally and inoculated, one plug per plate, onto water agar (1.5% weight/volume, wt/v) plates (60 × 15 mm) containing substrates for detecting targeted enzyme activity (classical test). The same seed cultures used to inoculate the agar plates were macerated in 70 mL sterile deionized water and used at 10% concentration (volume/volume, v/v) to inoculate malt extract liquid medium containing 2% (wt/v) malt extract, 2% (wt/v) glucose, and 0.1% (wt/v) bacto peptone. The liquid cultures were incubated on a rotary shaker (100 rpm) under conditions similar to those used for the agar-enzyme screening plates. To assay the enzymes, aliquots of liquid cultures were removed and filtered using a glass fiber filter paper (Whatman #934-AH) and applied to glass fiber filter discs (Whatman 7-mm diameter, 1.5 μm pore size) placed on agar plates (100 × 100 × 15 mm) containing the enzyme (RAPSA). Filtrates from all of the 14 test fungi, plus liquid medium, placed on a disc for control, were screened in the same plate for each of the enzymes tested. The screening plates for both the classical and the RAPSA test plates were wrapped with parafilm and incubated in a humid chamber under the following conditions: 1) room temperature (RT) overnight; 2) RT overnight plus 1 h at 50°C; 3) 37°C for 30 min; 4) 37°C overnight; 5) 37°C overnight plus 1 h at 50°C and 6) 50°C for 1 h.

The agar plugs from the classical method and the filter discs from the RAPSA were removed to evaluate substrate degradation by the enzyme. Congo red (0.1%, wt/v) complexing with the unhydrolyzed substrates was used to visualize the degradation zone of enzyme activity in most cases. Other reagents used for visualization included iodine for starch, ethanol for pustulan, cetylamethylammonium bromide for pectin, and o-dianisidine for glucose

from cellobiose. For lipase activity, calcium salts, crystallized in the solid medium, were clearly visible as opaque halos using a binocular microscope at low magnification.

Enzyme activity was rated based on the size of the zone of degradation visible. Each rating was based on three replicates.

Effect of nutrient source on enzyme profiles

The 14 test fungi were grown under three nutrient regimes in 50 ml of medium in 250-ml Erlenmeyer flasks for 3 weeks: 1) malt extract (2%, wt/v) medium with glucose (2%, wt/v) (ME + GLU); 2) defined liquid medium containing (SM) 2% (wt/v) hemlock sawdust (SD, SM + SD) or 3) defined liquid medium (SM) containing crude cell wall (CW, SM + CW) of the sapstain fungus *O. piceae* 387I. The defined liquid medium consisted of 0.2 g $MgSO_4 \cdot 7H_2O$, 0.9 g K_2HPO_4, 0.2 g KCl, 1 g NH_4NO_3, 0.002 g $FeSO_4 \cdot 7H_2O$, 0.02 g $ZnSO_4 \cdot 7H_2O$ and 0.002 g $MnCl_2 \cdot 4H_2O$ in 1 L distilled water (Ridout et al. 1988). Cell wall of *O. piceae* 387I was prepared by a modified method of Hearn and McKenzie (1980). The RAPSAs were carried out as described above.

RESULTS AND DISCUSSION

Comparison of enzymatic activities using mycelial plugs or culture filtrates

Table 1 compares extracellular enzyme activity detected using the classical method where agar plugs inoculated with the test fungus are placed on agar containing the test substrate with a Rapid Screening Assay (RAPSA) where filtrates from fungal liquid cultures are absorbed on glass filter discs, placed on agar containing the test substrate. Greater activity was observed by the majority of the fungi tested when screened using culture filtrates. Activity of β-galactomannanase was detected in the culture filtrates of *G. roseum* 784A, *G. roseum* 321A, and *G. viride* 623E but not in the plates with MA plugs. Also, *G. virens* 258C and *G. virens* 258D showed presence of β-1,6 glucanases and β-1,3 glucanase activity in the filtrates but not with the MA plug-inoculated plates. Among the reasons that might account for better elicitation of ECE activity in the filtrates of most test fungi is the fact that when the actively growing fungus is placed directly on the substrate, active fungal growth may predominate over enzyme production. Further, mycelial growth, occurring on the agar surface with this method, may interfere with visualization of enzymatic activity. Using the MA plug, chitinase activity was detected for only two biological control agents (*G. roseum* 321M and *G. viride* 258C, Table 1). Protease activity, however, was more pronounced in the MA plug inoculated plates and was produced by all fungi tested.

Effect of pH and buffer systems

The pH and buffer system requirements for assaying activity were examined. Table 2 summarizes the optimized conditions using the RAPSA for each enzyme tested. The most commonly used buffer systems, MES [2-N(morpholino)ethanesulfonic acid] and acetate, with pH ranging between 5–7 were tested. No difference in elicitation of ECE activity was observed. In contrast to the findings of Wood and Weisz (1987), the incorporation of MES in the agar screening plates interfered with the use of congo red in visualizing zones of degraded target substrates. The substrates under the filtrate soaked discs were often stained a darker purplish-red after the acetic acid wash in the congo red staining procedure. Although intensity of congo red staining is pH-dependent, the interference persisted even at higher pH. Thus to avoid inaccurate assessment of extracellular activity, the use of a sodium acetate buffer is recommended. The activity of most enzymes tested was found to be similar in the pH 5–7 range except for β-1,6-glucanase, chitinase, lipase, and protease where pH 5 was better. For assay of lipase activity, crystals of lauric acid and calcium salts were observed only in agar screening plates with the Tween 20 substrate when prepared in acetate buffer at pH 5. No lipase ac-

TABLE 1. *Screening of extracellular enzyme activity[1] on agar plates inoculated with agar plugs or culture filtrates.*

Enzyme	Substrate	[2]Assay	Test fungus[3] ME 386 E	TP 228 B	TV 161 1	GR 784 A	GR 321 A	GR 321 M	GS 810 A	GV 623 E	GV 258 C	GV 258 D	A A 2H	AP 132 Q	OP 387 1
Cellulases	Avicel	P	±					+							
		F							±						
β-1,4-Endoglucanase	CMC	P				+	+	+	2+	+	3+	+	2+		2+
		F			±	2+	+	±	+	2+	±	+	2+		+
β-1,6-Glucanase	Pustulan	P	3+			+	+	3+	±	2+				±	+
		F	3+	3+	3+	2+	+	2+	2+	2+	2+	3+	+	±	3+
β-1,3-Glucanase	Laminarin	P	±					+	+						
		F		±					+		+	+			+
α-1,3-Mannanase	α-Mannan	P													
		F						±							
β-1,4-Xylanase	Xylan	P				+	±	2+	2+	±	+	+	±		2+
		F				±	±	+	+	±	±	±	±	±	2+
β-1,4-Galactomannanase	Locust bean	P	±					±	±		±	+			2+
		F	±			+	+	+	+	+					+
β-1,4-Glucomannanase	Konjac root	P						±					±	±	2+
		F				+	+	±	±	+	+	+	2+	2+	2+
Proteases	Skim milk powder	P	+	+	+	2+	±	2+	2+	+	+	+	+	+	2+
		F	±	±	±	2+	+	+	+	±	±	±	+	+	+
Chitinase	Chitin	P						+			+				±
		F	±	±	±	±		±		±	±	±	±	±	±

[1] Extracellular enzyme activity rating: ± = No distinct clearing zone but the color of stained zone was much lighter where the plug filter was placed. + = Definite clearing zone. +2 = Clearing larger than area of disc. +3 = Clearing zone larger than +2.

[2] P: Screening carried out using agar plugs covered with actively growing mycelium of the test fungus. [3] F: Screening carried out using glass fiber discs saturated with culture filtrate from the actively growing fungus.

[3] ME386E = *Mariannaea elegans* 386E, TP228B = *Trichoderma pseudokoningii* 228B, TV1611 = T. viride 1611, GR784A = *Gliocladium roseum* 784A, GR321A = *G. roseum* 321A, GR321M = *G. roseum* 321M, GS810A = *G. solani* 810A, GV623E = *G. viride*, GV258C = *G. viride* 258C, GV258D = *G. virens* 258D.

tivity was observed when glycerol tributyrate was used as substrate, at any pH tested.

Other factors affecting the assay

Generally, increasing substrate concentration from 0.15% to 0.5% increased contrast in the visualization step (Table 2). However, varying incubation time and temperature greatly affected the degree of extracellular enzyme activity elicited. Optimum incubation time and temperature for most substrates were room temperature overnight plus 1 h at 50°C. Exceptions were avicel, cellulose azure, and chitin where 37°C was optimum. These conditions allowed detection of the lowest enzyme activity while controlling the diffusion of activity from very active enzymes and interference with neighboring samples on the same screening plate.

Activities of α-1,3-mannanase and β-1,3-glucanase were assayed using laminarin and pachyman, respectively. Congo red staining of undegraded laminarin required prolonged staining at 37°C for visualization of the degraded zone. Laminarin did not complex well with congo red at room temperature, but prolonged staining at elevated temperature followed by a brief rinse in sodium chloride revealed a positive response. In situations where glucose was absent from the medium, an alternate reagent, 2,3,5-triphenyltetrazolium chloride, was used. This reagent stained the degradation product (glucose) instead of the undegraded substrate. Although pachyman was insoluble, congo red staining of the undegraded substrate was applicable to reveal ECE activity in this substrate. None of the visualization techniques and incubation conditions tested detected ECE activity on the pullulan substrate. β-1,6-glucanase activity on pustulan was visualized using ethanol precipitation of the undegraded substrate and efficiency of detection was optimum at pH of 5.

Effect of nutrient source on enzyme profiles

Examples of the RAPSA tests for several enzyme systems, using the test three media, are shown in Fig. 1. The detection of ECE activity varied with the substrate and with the nutrient source. For example, β-1,3-glucanase production by both *G. roseum* 784A and *G. roseum* 321M was detected on pachyman but not laminarin in both glucose and sawdust media, suggesting that substrate specific β-1,3-glucanases may be produced by various fungi. Activity of β-1,3-glucanase was generally lower in glucose-amended medium than that observed in sawdust or cell-wall medium. The observation of enhanced induced β-1,3-glucanase activity under stress conditions of limited nutrient availability over the constitutively produced β-1,3-glucanase in an enriched environment supports the observation by Pitson et al. (1991) and Del Ray et al. (1979) that β-1,3-glucanase can be cell wall bound, cytoplasmic or exocellular, and many fungi appeared to have these enzymes repressed by catabolites such as glucose.

The enzymes most frequently produced by the candidate fungi, irrespective of medium, were xylanase and cellulase, suggesting that these enzymes are intrinsically produced. As expected, most cellulase and β-mannanase activities were induced in cultures with wood as growth substrate. Cellobiase activity was exhibited only where ECE activity was assayed on cellulose azure. Activity on this substrate was detected by the presence of endo- and exoglucanases (cellobiohydrolase). The latter enzyme produces cellobiose, which necessitates the production of cellobiase in the system for effective cellulose hydrolysis.

Most fungi tested demonstrated the highest extracellular enzyme activity with glucose in the medium. Less activity was detected with sawdust, while very little activity was observed with cell wall of *O. piceae,* indicating that most of the ECE activity can be constitutively produced in an enriched medium while little can be induced. Lipase activity was observed only in glucose amended medium and elicited only by a few candidate fungi including *G. roseum* 321M, *G. viride* 623E, *G. virens* 258D, the sapstain fungus, *A. pullulans*

TABLE 2. *Optimized conditions for the detection of extracellular enzymes using the Rapid Agar Plate Screening Assay and visualization of positive responses.*

Enzyme	Substrate and concentration	pH	Incubation conditions	Reagents for visualization	Positive response
α-1,3-mannanase	Yeast α-mannan	5–7	[1]RT, O/N + 50°C, 1 h	Congo red complexing agent for substrate	Pale red to clear zone formed
β-glucanase					
β-1,3-glucanase	Laminaran, 0.5;	5–7	RT, O/N + 50°C, 1 h	Congo red complexing agent for substrate, or 2,3,5-triphenyltetrazolium chloride (staining degradation product)	Pale red to clear zone formed
	Pachyman, 0.5	5–7	RT, O/N + 50°C, 1 h	Congo red complexing agent for substrate	Pale red to clear zone formed
β-1,6-glucanase	Pustulan, 0.5	5	RT, O/N + 50°C, 1 h	95% ethanol (enhanced contrast)	Pale red zone formed
Chitinase	Chitin, 0.5	5	[2]37°C, O/N, + 50°C, 1 h	Congo red complexing agent for substrate	Pale red to clear zone formed
Cellulase					
Cellulases	Avicel PH101, 0.5	5–7	37°C, O/N, + 50°C, 1 h	Congo red complexing agent for substrate	Pale red to clear zone formed
	Cellulose azure				Pale blue degradation zone against dark blue insoluble dyed substrate
β-1,4-endoglucanase	Carboxymethylcellulose, 0.25	5–7	RT, O/N + 50°C, 1 h	Congo red complexing agent for substrate	Pale red to clear zone formed
β-1,4-glucosidase	Cellobiose, 20 mM	5–7	[3]RT, O/N	PGO (glucose and peroxidase) enzyme + *o*-dianisidine staining of the glucose	Brown zone formed
Hemicellulase					
β-1,4-galactomannase	Locust bean gum, 0.5	5–7	RT, O/N + 50°C	Congo red complexing agent for substrate	Pale red to clear zone formed
β-1,4-glucomannase	Konjac root powder, 0.5	5–7	RT, O/N + 50°C	Congo red complexing agent for substrate	Pale red to clear zone formed
β-1,4-xylanase	Oat spelts xylan, 0.5	5–7	RT, O/N + 50°C	Congo red complexing agent for substrate	Pale red to clear zone formed
Lipase	Sorbitan monolaurate, Tween 20, 1% v/v	5	RT, O/N + 50°C	No reagent used	Opaque zone formed against clear background
				Calcium salt crystals visible using 50× magnification	

TABLE 2. *Continued.*

Enzyme	Substrate and concentration	pH	Incubation conditions	Reagents for visualization	Positive response
Pectinase	Apple pectin, 0.5	5–7	RT, O/N + 50°C	Cetylmethylammonium bromide	Clear zone formed in precipitated substrate
Proteolytic enzymes	Skim milk powder, 1.0	5	RT, O/N + 50°C	Congo red complexing agent for substrate	Pale red to clear zone formed
Starch hydrolase, amylase	Potato soluble starch, 0.5	5–7	RT, O/N + 50°C	Iodine solution	Clear zone formed against stained background

[1] Incubated overnight at room temperature plus an additional hour at 50°C.
[2] Incubated overnight at 37°C plus an additional hour at 50°C.
[3] Incubated room temperature, overnight.

132Q and the control fungus *T. harzianum* 160M.

Selection of potential biological control agents

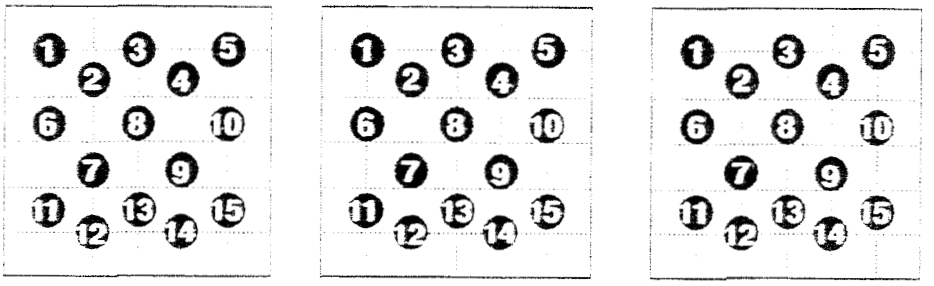
On the basis of ECE activity observed when fungi were grown in the presence of the three nutrient sources used in this study, three groups of fungi were recognized (Tables 3a, b, c). Group I fungi (Table 3a) produced the greatest enzyme activity with glucose. Group II fungi (Table 3b) produced similar enzyme activity in glucose and sawdust-amended media, while those fungi from Group III fungi (Table 3c) showed the capacity to produce extracellular enzymes in the presence of sawdust or cell wall while enzyme activity on glucose was variable.

Group I fungi, demonstrating the highest enzyme activity in the presence of glucose, included *M. elegans* 386E, *T. pseudokoningii* 228B, *T. viride* 161I, *G. roseum* 321A, *G. solani* 810A, and the sapstain fungi *A. pullulans* 132Q and *O. piceae* 387I. The most common enzymes observed for these fungi were chitinase, β-1,4-xylanase and/or amylase.

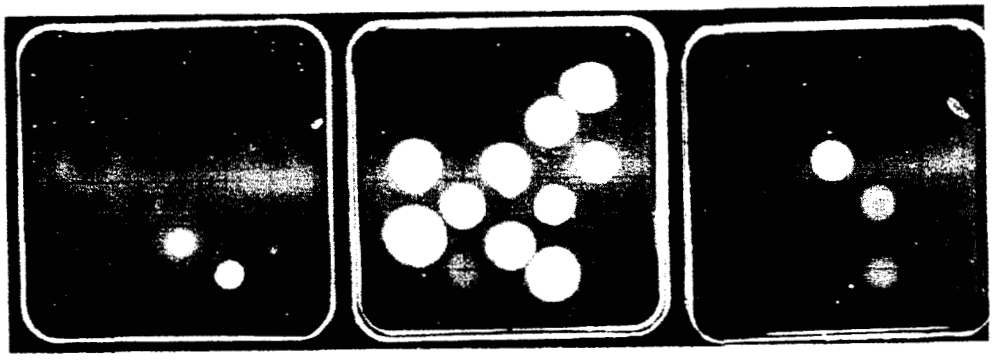
Both *M. elegans* 386E and *G. solani* 810A were weak producers of extracellular enzymes. While *M. elegans* 386E produced endo-glucanase, β-glucanases, xylanase, α-mannanase, and chitinase on glucose amended media, *G. solani* 810A only produced protease. If extracellular enzyme production is important in biological control mechanisms, then

→

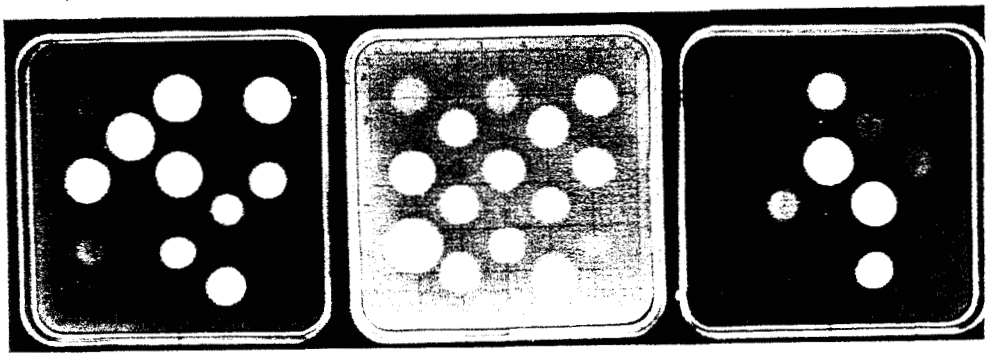
FIG. 1. Rapid agar plate screening assay for extracellular enzyme activity testing culture filtrates from fungi grown in a defined liquid media (SM) in the presence of glucose (ME), sawdust (SD), and fungal cell wall (CW). Test fungi: 1 = *Mariannaea elegans,* 2 = *Trichoderma pseudokoningii* 228B, 3 = *T. viride* 161I, 4 = *Gliocladium roseum* 784A, 5 = *G. roseum* 321A, 6 = *G. roseum* 321M, 7 = *G. solani* 810A, 8 = *G. viride,* 9 = *G. viride* 258C, 10 = *G. virens* 258D, 11 = *A. alternata* 2H, 12 = *A. pullulans,* 13 = *O. piceae,* 14 = *T. harzianum,* 15 = Medium control.



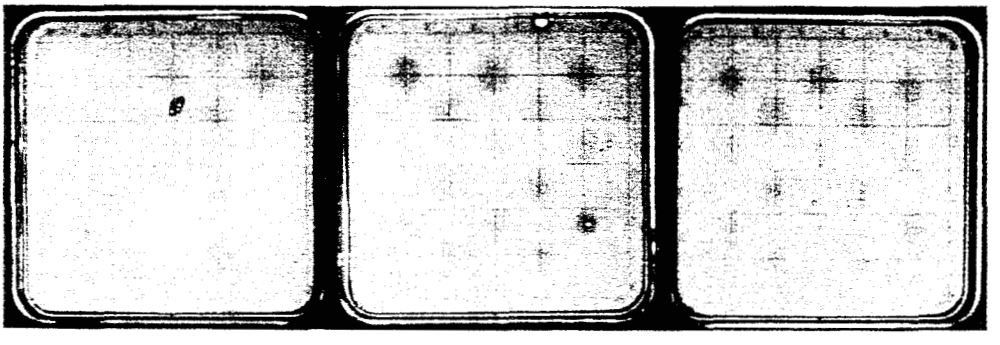
ME- GLU SM-SD SM+CW



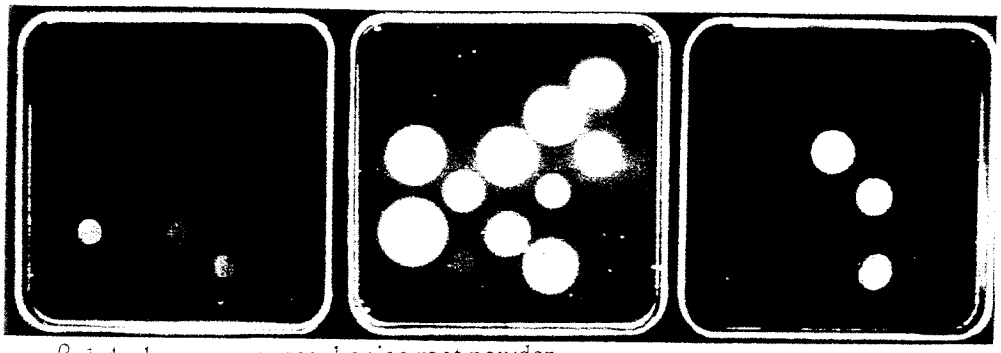
β-1,4-galactomannanase, Locust bean gum



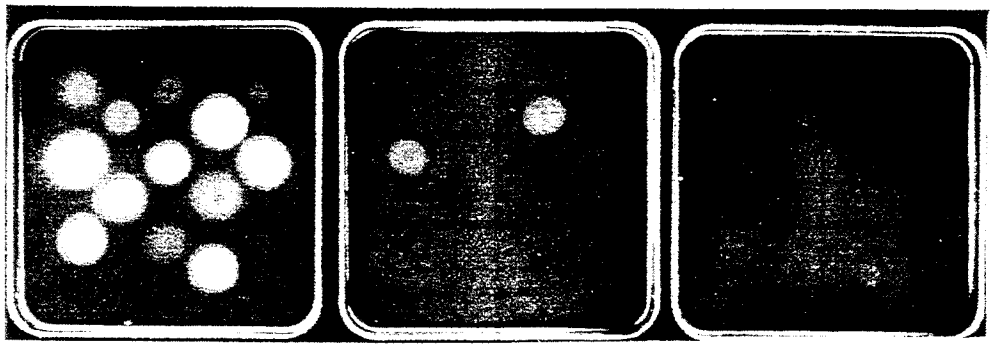
Amylase, starch



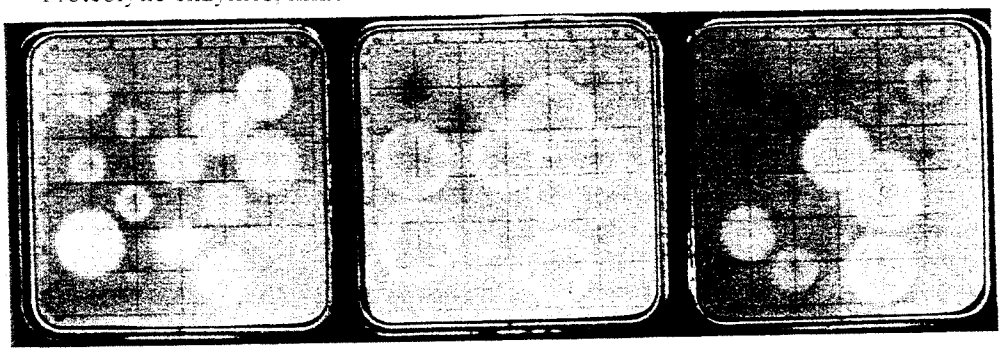
Cellulases, avicel



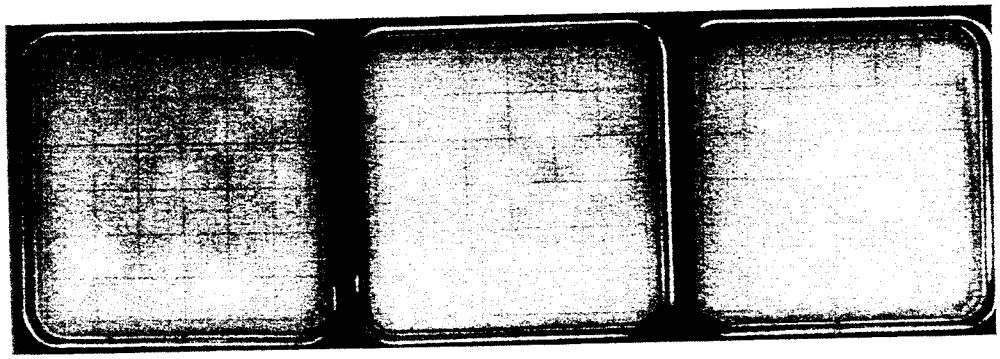
β-1,4-glucomannanase, konjac root powder



Proteolytic enzymes, milk



β-1,4-endoglucanase, carboxymethylcellulose (CMC)



Cellulases, cellulose azure

TABLE 3A. *Extracellular enzyme activity of potential biological control fungi and sapstain fungi belonging to Group I fungi.*

	Fungus	Enzyme	Substrate
Group I: Fungi producing the greatest enzyme activity[1] with glucose	*M. elegans* 386E	β-1,4-Xylanase	Oats spelts xylan
		β-1,3-Glucanase	Laminarin
		β-1,6-Glucanase	Pustulan
	T. pseudokoningii 228B	β-1,4-Xylanase	Oats spelts xylan
		β-1,6-Glucanase	Pustulan
		Chitinase	Chitin
		Amylase	Potato soluble starch
	T. viride 1611	β-1,4-Xylanase	Oats spelts xylan
		β-1,3-Glucanase	Laminarin
		β-1,6-Glucanase	Pustulan
		Amylase	Potato soluble starch
	G. roseum 321A	β-1,4-Endoglucanase	CMC
		β-1,4-Xylanase	Oats spelts xylan
		α-1,3-Mannanase	Yeast α-mannan
		Chitinase	Chitin
		Amylase	Potato soluble starch
	G. solani 810A	Proteases	Skim milk powder
	A. pullulans 132Q	β-1,4-Endoglucanase	CMC
		Pectinase	Apple pectin
	O. piceae 3871	β-1,4-Xylanase	Oats spelts xylan
		α-1,3-Mannanase	Yeast α-mannan
		β-1,3-Glucanase	Pachyman
		β-1,6-Glucanase	Pustulan

[1] Extracellular enzyme activity rated as +2 (clearing zone larger than area of disc) and +3 (clearing zone larger than +2) only were considered. All ECE activities were determined by RAPSA.

M. elegans 386E and *G. solani* 810A showed the least potential.

Group II fungi (Table 3b), including *G. roseum* 784A, *G. roseum* 321M, *G. virens* 258D, and the sapstain fungus *A. alternata* 2H, exhibited the highest ECE activity on both the glucose and the sawdust amended media. The elicitation of the full complement of cellulases and hemicellulase on sawdust indicates the capacity to utilize some of the major components of wood. *G. roseum* 784A produced the highest activity and widest range of enzymes of all the fungi in this group and demonstrated β-1,4-xylanase activity on the cell-wall medium. Both *G. roseum* 784A and *G. roseum* 321M produced protease on sawdust medium. Group II thus demonstrated the ability to utilize more complex and not easily accessible substrates that represent major components of wood. Contrary to expectation, all three fungi elicited strong chitinase activity in the presence of sawdust but not cell wall.

Group III fungi, the most active ECE producers in all three media types (Table 3c), included *G. viride* 623E, *G. virens* 258C, and the experimental control fungus, *T. harzianum* 160M. They produced the full complement of cellulases, hemicellulases, and β-1,3- and 1,6-glucanases as well as chitinase and/or amylase on both sawdust and cell-wall-amended media. *T. harzianum* elicited the highest enzymatic activities, notably cellulases, xylanase, α-mannase, β-glucanase, and chitinase on all three media used in the study. Protease activity was weak with cell wall and glucose and not detected with sawdust media. The high enzyme activity exhibited by Group III fungi is an indication of secondary resource capability. If primary and secondary resource capture capabilities are required attributes for a successful biological control fungus, then *G. viride* 623E and *G. virens* 258C demonstrate the greatest potential as biological control agents.

Of all ten biological control fungi screened,

TABLE 3B. *Extracellular enzyme activity of potential biological control fungi and sapstain fungi belonging to Group II fungi.*

	Fungus	Enzyme	Substrate
Group II: Fungi producing similar enzyme activity[1] in glucose and sawdust amended media	*G. virens* 258D	β-1,4-Endoglucanase	CMC
		β-1,4-Xylanase	Oats spelts xylan
		α-1,3-Mannanase	Yeast α-mannan
		β-1,3-Glucanase	Laminarin
		β-1,3-Glucanase	Pachyman
		Chitinase	Chitin
		Proteases	Skim milk powder
		Lipase	Tween 20
	G. roseum 321M	β-1,4-Endoglucanase	CMC
		β-1,4-Galactomannanase	Locust bean gum
		β-1,4-Glucomannanase	Konjac root
		β-1,4-Xylanase	Oats spelts xylan
		α-1,3-Mannanase	Yeast α-mannan
		Chitinase	Chitin
		Proteases	Skim milk powder
		Pectinase	Apple pectin
		Amylase	Potato soluble starch
	G. roseum 784A	β-1,4-Endoglucanase	CMC
		β-1,4-Galactomannanase	Locust bean gum
		β-1,4-Glucomannanase	Konjac root
		β-1,4-Xylanase	Oats spelts xylan
		α-1,3-Mannanase	Yeast α-mannan
		β-1,3-Glucanase	Pachyman
		β-1,6-Glucanase	Pustulan
		Chitinase	Chitin
		Proteases	Skim milk powder
		Pectinase	Apple pectin
	A. alternata 2H	β-1,4-Endoglucanase	CMC
		β-1,4-Galactomannanase	Locust bean gum
		β-1,4-Glucomannanase	Konjac root
		β-1,4-Xylanase	Oats spelts xylan
		α-1,3-Mannanase	Yeast α-mannan
		Chitinase	Chitin
		Proteases	Skim milk powder

[1] Extracellular enzyme activity rated as +2 (clearing zone larger than area of disc) and +3 (clearing zone larger than +2) only were considered. All ECE activities were determined by RAPSA.

G. viride 623E elicited the greatest ECE activity from filtrates of all three media. In addition to chitinase and β-glucanase activities, it exhibited significant cellulase and hemicellulase activity in the presence of CW. It also demonstrated the full spectrum ECE activity in both CW and SD, an indication that it is efficient at resource capture utilizing wood components and producing cell wall lytic enzymes.

CONCLUSIONS

The use of RAPSA permits simultaneous comparative screening of extracellular enzyme activity of up to 15 test organisms. In comparison with the classical method, the RAPSA demonstrated greater sensitivity in detecting enzymatic activity by the majority of fungi tested for all enzymes screened with the exception of chitinase. Although no differences in elicitation of extracellular enzymes were observed, the use of a sodium acetate buffer system is recommended because MES interfered with the use of congo red in visualizing zones of degraded target substrates.

The use of this assay to screen potential bioprotectants for wood indicated that *G. viride*

TABLE 3C. *Extracellular enzyme activity of potential biological control fungi and sapstain fungi belonging to Group III fungi.*

	Fungus	Enzyme	Substrate
Group III: Fungi producing similar enzyme activity[1] in sawdust and cell wall amended media but variable activity with glucose	*G. virens* 258C	β-1,4-Endoglucanase	CMC
		β-1,4-Xylanase	Oats spelts xylan
		α-1,3-Mannanase	Yeast α-mannan
		β-1,3-Glucanase	Pachyman
		Chitinase	Chitin
	G. viride 623E	β-1,4-Endoglucanase	CMC
		β-1,4-Xylanase	Oats spelts xylan
		β-1,6-Glucanase	Pustulan
		β-1,4-Galactomannanase	Locust bean gum
		β-1,4-Glucomannanase	Konjac root
		α-1,3-Mannanase	Yeast α-mannan
		β-1,3-Glucanase	Laminarin
		β-1,3-Glucanase	Pachyman
		Amylase	Potato soluble starch
		Chitinase	Chitin
	T. harzianum 160M	β-1,4-Xylanase	Oats spelts xylan
		Cellulases	Cellulose azure
		β-1,4-glucosidase	Cellobiose
		β-1,4-Galactomannanase	Locust bean gum
		α-1,3-Mannanase	Yeast α-mannan
		β-1,3-Glucanase	Laminarin
		β-1,6-Glucanase	Pustulan
		Chitinase	Chitin

[1] Extracellular enzyme activity rated as +2 (clearing zone larger than area of disc) and +3 (clearing zone larger than +2) only were considered. All ECE activities were determined by RAPSA.

623E demonstrated the greatest potential for resource capture and the production of cell-wall lytic enzymes, two necessary traits for successful bioprotection. Both *G. viride* 623E and *G. virens* 258C demonstrated high potential for both primary and secondary resource capture.

ACKNOWLEDGEMENT

This work conducted at Forintek Canada Corp. and was supported by the Canadian Forestry Service, Natural Resources Canada.

REFERENCES

ARONSON, J. M. 1965. The cell wall. *In* G. C. Ainsworth and A. S. Sussman, eds. The fungi, vol. I. The fungal cell. Academic Press. New York, NY.

DAWSON-ANDOH, B. E., AND J. J. MORRELL. 1997. Biological protection of freshly sawn sapwood from biological discolorations. Pages 3–9 *in* Prevention of discolorations in hardwoods and softwood logs and lumber. Forest Prod. Soc. Pub. No. 7283.

DEL RAY, F., I. GARCIA-ACHA, AND C. NOMBELA. 1979. The regulation of β-glucanase synthesis in fungi and yeast. J. Gen. Microb. 110:83–89.

DONLY, B. C., AND A. W. DAY. 1984. A survey of extracellular enzymes in the smut fungi. Bot. Gaz. 145:483–486.

FRAVEL, D. R. 1988. Role of antibiosis in the biocontrol of plant diseases. Ann. Rev. Phytopathol. 26:75–91.

HAGERMAN, A. E., D. M. BLAU, AND A. L. MCCLURE. 1985. Plate assay for determining the time of production of protease, cellulase, and pectinase by germinating fungal spores. Anal. Biochem. 151:334–342.

HANKIN, L., AND S. L. ANAGNOSTAKIS. 1975. The use of solid media for the detection of enzyme production by fungi. Mycologia 67:597–607.

HARNEY, S., AND P. WIDDEN. 1991. Physiological properties of the entomopathogenic hyphomycete *Paecilomyces farinosus* in relation to its role in the forest ecosystem. Can. J. Bot. 69:1–5.

HEARN, V. M. AND D. W. R. MACKENZIE. 1980. The preparation and partial purification of fractions from mycelial fungi with antigenic activity. Molecular Immun. 17: 1097–1103.

KJOLLER, A., AND S. STRUWE. 1980. Microfungi of decomposing red alder leaves and their substrate utilization. Soil Biol. Biochem. 12:425–431.

LEWIS, J. A., AND C. C. PAPAVIZAS. 1991. Biocontrol of

plant diseases: The approach for tomorrow. Crop Protect. 10:95–105.

LEWIS, K., J. M. WHIPPS, AND R. C. COOKE. 1989. Mechanisms of biological disease control with special reference to the case of *Pythium oligandrum* as an antagonist. Pages 191–217 *in* J. M. Whipps and R. D. Lumsden, eds. Biotechnology of fungi for improving plant growth. Cambridge University Press, UK.

LUMSDEN, R. D., AND J. A. LEWIS. 1989. Selection, production, formulation, and commercial use of plant disease biocontrol fungi: Problems and progress. Pages 170–190 *in* J. M. Whipps and R. D. Lumsden, eds. Biotechnology of fungi for improving plant growth. Cambridge University Press, UK.

LYNCH, J. M. 1990. Fungi as antagonists. Pages 243–253 *in* New Directions in biological control: Alternatives for suppressing agricultural pests and diseases. Alan R. Liss, Inc.

MCAFEE, B. J., AND M. M. GIGNAC. 1997. Biological control of fungal growth on unseasoned lumber following pasteurization. Mater. und Org. 31:45–61.

PAN, S. W., X. S. YE, AND J. KUC. 1991. A technique for detection of chitinase, β-1,3-glucanases and protein pattern after a single separation using polyacrylamide gel electrophoresis of IEF. Phytopathol. 81:970–974.

PAPAVIZAS, G. C. 1985. *Trichoderma* and *Gliocladium*: Biology, ecology, and potential for biocontrol. Ann. Rev. Phytopathol. 23:23–54.

PITSON, S. R., J. SEVIOUR, J. BOTT, AND S. J. STANSINOPOULOS. 1991. Production of β-glucanases in *Acremonium* and *Cephalosporium* isolates. Mycol. Res. 95: 352–356.

RAYNER, A. D. M., AND N. K. TODD. 1979. Population and community structure and dynamics of fungi in decaying wood. Advan. Botan. Res. 7:333–420.

RIDOUT, C. J., J. R. COLEY-SMITH, AND J. M. LYNCH. 1988. Fracionation of extra-cellular enzymes from a mycoparasitic strain of *Trichoderma harzianum*. Enzyme Microb. Technol. 10:180–186.

SAKSIRIRAT, W., AND H. H. HOPE. 1991. Secretion of extracellular enzymes by *Verticillium psalliotae* Treschow and *Verticillium lecanii* (Zimm.) Viegas during growth on uredospores of soybean rust fungus (*Phakospora pachyrhzi* Syd.) in liquid cultures. J. Phytopathol. 131: 161–173.

VILLANUEVA, J. R. 1966. The protoplast. *in* C. G. Ainsworth and A. S. Sussman, eds. The fungi, vol. II. The fungal organism. Academic Press. New York and London.

WOOD, P. J., AND J. WEISZ. 1987. Detection and assay of (1-4)-β-D-Glucanase, (1-3)(1-4)-β-D-Glucanase and xylanase based complex of substrate with congo red. Cereal Chem. 64:8–15.

YANG, D. Q., AND L. ROSSIGNOL. 1999. Evaluation of *Gliocladium roseum* against wood-degrading fungi in vitro and on major Canadian wood species. Biocontrol Sci. Technol. 9:409–420.