J Wood Sci (2004) 50:445–449
DOI 10.1007/s10086-003-0587-x

NOTE

Ying Xiao · Jeffrey J. Morrell

Production of protoplasts from cultures of *Ophiostoma picea*

Received: January 8, 2003 / Accepted: August 4, 2003

Abstract A method is described for producing viable protoplasts from germinating conidia (germlings) of *Ophiostoma picea*. The use of $MgSO_4$-based osmotic stabilizers significantly improved protoplast release, as did the use of 20-h-old germlings. Protoplast release rates also increased with higher enzyme concentration and incubation times, but there were associated negative effects, including reduced regeneration. The results indicate that the use of young germlings, low lytic enzyme levels, and short exposure periods produced the most viable protoplasts.

Key words *Ophiostoma picea* · Bluestain · Protoplasts · Biological control

Introduction

Discoloration of lumber by fungi is a major cause of economic loss for wood processors. From the time it is cut in the forest until it dries to below 20% to 25% moisture content – and later, if it is inadvertently rewetted – wood can be rapidly colonized by a diverse array of fungi that can discolor the surface via the production of pigmented spores or the interior through melanized hyphae in the cell lumens. Although the precise costs that result from fungal discoloration are difficult to calculate, it has been estimated that they cause approximately US$10 million in losses per year.[1] Surveys of lumber producers in the western United States suggest that this figure is extremely conservative.[2] Among the most important fungi causing discoloration of lumber are the *Ophiostoma* species. Many of these species are widely distributed and cause deep discoloration of freshly sawn wood. *Ophiostoma picea* is an economically important fungus in many coniferous species in western North America.

Discoloration of lumber has generally been controlled by drying the wood below 20% or, where that was not feasible, applying surface coatings of prophylactic fungicides. Most mills lack the kiln capacity to dry all their production; however, there are some applications for which the user does not desire dry wood. Chemicals carry with them the risk of worker exposure and the potential for scrutiny by an increasingly chemophobic public.

One alternative to chemical treatment is biological control (biocontrol): using one organism to control the detrimental activities of another. Biocontrol is not a new concept; fungi have been employed for this purpose on a variety of wood-based materials,[3–5] but there have been few broadly successful applications against stain and mold fungi.[6–8] Failures to achieve successful biocontrol have been attributed to the inability of the biocontrol organism to function under field conditions, where growth is limited by competing organisms and environmental conditions may be less than ideal. The failures have highlighted the need for more fundamental research on in situ microbial interactions between biocontrol agents and their intended targets.

An important aspect of this need is the ability to study microbial interactions within wood. This is generally difficult when the biocontrol agent is a fungus, because most of the hyphae of both the stain fungi and the biocontrol agent are hyaline, making it difficult to determine which fungi are present. A number of fluorescent probes are available that are specific for components in fungal hyphae, but most of these react with hyphal components common to many fungi.[9]

One approach to this dilemma is to transform one of the test fungi by inserting genetic material capable of producing fluorescent proteins that are continuously expressed. This process would allow visualization of all of the hyphae of that species in wood by using fluorescence microscopy. An important first step in this transformation is to develop systems for eliminating the fungal cell wall so that specific probes can be inserted into the cells. The simplest method for accomplishing this task is to generate fungal protoplasts.

Y. Xiao (✉) · J.J. Morrell
Department of Wood Science and Engineering, Oregon State University, Corvallis, OR 97331, USA
Tel. +1-541-737-4302; Fax +1-541-737-3385
e-mail: shelly.xiao@orst.edu

Protoplasts are the wall-less states of cells after the cell walls have been removed through enzymatic digestion. Many factors including type and concentration of enzymes, treating time, type of osmotic stabilizers, fungal species, and culture age can have pronounced effects on the yield of protoplasts and their regeneration.[10] A number of protocols on protoplasting from some wood-inhabiting fungi have been reported.[11–18] The conditions for protoplasting varied widely in these studies, suggesting the need to define optimal conditions for protoplasting from each species. There are no reports of procedures for generating protoplasts from *O. picea*. In this report, we outline procedures for producing protoplasts of *O. picea* as a prelude to transformation.

Materials and methods

Cultures of *Ophiostoma picea* (Isolate AU135-1, Forintek, Canada Corp.) were maintained on 1% malt extract agar. The strain was originally isolated from white spruce lumber in British Columbia, Canada. Prior to use, the fungus was inoculated on an agar medium containing 1.0% malt extract, 1.0% glucose, 0.1% yeast extract, and 1.5% agar (MGY agar), and incubated for 12 days at 25°C in darkness. The test fungus produced abundant conidia on this medium in preliminary tests. Conidia forming on the agar surface were gently washed from the plates using sterile distilled water. Conidia were used either directly for protoplasting or were used to produce germlings. Conidia for direct use were collected by centrifugation (7500*g* for 5 min). Conidia were washed twice with distilled water and then once with one of two osmotic stabilizers, either 0.5 M $MgSO_4$ or 0.5 M mannitol in 50 mM maleic-NaOH (pH 5.5).[13] Conidia were then transferred to a sterile glass tube (16 × 30 mm).

Germlings were produced by adding approximately 1×10^8 conidia into 100 ml of a medium containing 2% potato dextrose and 0.1% malt extract (PDB Broth). The broth was incubated for 16, 20, or 24 h on a rotary shaker (100 rpm) at room temperature (20°–23°C) to produce germlings. Germlings were collected by filtration through a 20-μm nylon mesh (Sefar America, Depew, NY). The germlings were washed three times with sterile distilled water and then three times with osmotic stabilizer, either 0.5 M $MgSO_4$ or 0.5 M mannitol in 50 mM maleic-NaOH (pH 5.5). Wet germlings were then transferred to a sterile glass tube (16 × 300 mm).

Protoplast production

Lyophilized lysing enzyme produced by *Trichoderma harzianum* (Sigma Chemicals, St. Louis, MO) was dissolved in the appropriate osmotic stabilizer at 0.4, 0.7, or 1.0% (w/w). The same osmotic stabilizer was used throughout all procedures for a single treatment. The resulting solution was sterilized by filtration through a 0.22-μm membrane.

Ten milliliters of lysing enzyme was added to test tubes containing conidia or germlings. The tubes were incubated for 30, 60, or 120 min on a nutator (Clay Adams, Sparks, MD) at 25°C. The solution was diluted to 20–25 ml with the appropriate osmotic stabilizer, and filtered through 10-μm nylon mesh. The filtered solution was centrifuged at 7500*g* for 10 min and the supernatant was decanted and discarded. The pellet was resuspended in 20 ml of the appropriate osmotic stabilizer and centrifuged again. After resuspension, protoplasts and hyphal fragments were counted with a hemacytometer.

There were three replicates for each trial. For each replicate, 1 ml of protoplast solution containing approximately 1×10^7 cells received 100 μl of a solution containing 50 μg/ml of fluorescent isothiocyanate (FITC)-coupled wheat germ agglutinin (WGA).[9,19] The solution was incubated in darkness for 30 min and the cells were collected by centrifugation (2500*g* for 5 min), rinsed with 1 ml of the appropriate osmotic stabilizer, and centrifuged. The resulting cells were observed with a Nikon Elipse E400 microscope equipped with a filter specific for FITC, and the numbers of fluorescent and total protoplasts were counted.

Protoplasts were produced from conidia by adding 10 ml of lysing enzyme solution to washed conidia in the appropriate osmotic stabilizers. These tubes were incubated on the nutator shaker for 2, 4, or 24 h at room temperature. Each variable was investigated on three replicates. Protoplast production was assessed as described above.

Protoplast regeneration

Although it is important to be able to remove cell walls, fungal protoplasts can only remain in this state for relatively short periods and must eventually be allowed to regenerate cell walls. The use of harsh conditions can adversely affect the ability of the protoplast to regenerate. Protoplast regeneration was assessed by adding 1 ml of protoplasts that had previously been diluted to 1×10^4 protoplasts/ml in the appropriate osmotic buffer into 9 ml of molten MGY medium containing 1.2 M sorbitol. This mixture was placed in a plastic petri dish and incubated for 4 days at room temperature. Regeneration was then assessed by counting the colony-forming units. The percentage of the colony-forming units produced from the total cells in the dilution was defined as the regeneration rate.

Data analysis

The effects of enzyme concentration, osmotic stabilizer, and treatment time were assessed using multiple linear regression, and treatment measures were compared using Fisher's least significant difference test on SAS (SAS ver 7.0, SAS Institute, Cary, NC).

Results and discussion

Protoplasts could not be produced from conidia, even when conidia were exposed to the lytic enzyme for 24 h. This

Table 1. Number of protoplasts produced from germlings exposed to 0.4% to 1.0% lytic enzyme in $MgSO_4$-maleic-NaOH or mannitol-maleic-NaOH stabilizer for 1 h

Stabilizer	Enzyme concentration (%)		
	0.4	0.7	1.0
$MgSO_4$-maleic-NaOH	22.13 (0.46)[a]	27.77 (12.49)	28.48 (2.12)
Mannitol-maleic-NaOH	0.79 (0.12)	1.55 (0.09)	3.15 (0.39)

[a]Number of protoplasts $\times 10^7$/ml. Values represent means of 3 replicates with standard deviations in parentheses

suggests that cell wall components in the conidia were less susceptible to the chitinase systems produced by *Trichoderma harzianum*. As a result, all data reported are for germlings.

Effects of buffer on protoplast production

Protoplast levels were significantly greater ($P < 0.001$) when $MgSO_4$ was incorporated into the osmotic stabilizer. Average protoplast production ranged from 0.79 to 3.15 $\times$ 10^7 protoplasts/ml when mannitol was used, compared with 22.13 to 28.48 $\times$ 10^7 protoplasts/ml in $MgSO_4$ (Table 1). Protoplast levels also tended to increase steadily with increasing enzyme concentration, suggesting that the high concentrations of lytic enzymes enhanced cell wall removal (Table 1).

These preliminary trials indicated that the $MgSO_4$-based osmotic stabilizer produced more protoplasts than did mannitol; this stabilizer was therefore used in all subsequent tests.

Effects of germling age on protoplast production

Wet mycelial mass tended to increase with incubation period (Fig. 1); however, protoplast production peaked after 20 h of incubation and was significantly higher at that point than after 16 or 24 h ($p < 0.001$) (Fig. 2). These results suggest that early stages of mycelial growth may be most amenable to protoplasting. Hyphal thickening and senescing away from the growing tip may reduce the potential for protoplast production. Conversely, the presence of hyphal fragments tended to steadily decline as germling age increased, although the differences were not significant. The presence of high levels of hyphal fragments in the germling suspension is undesirable because they consume enzyme and suggest the presence of nonviable hyphal fragments that could not release protoplasts. Incubation for 20 or 24 h appeared to markedly reduce the levels of hyphal fragments.

Enzyme concentration and treatment time both significantly affected protoplast release ($p < 0.0001$ and $p = 0.0002$, respectively; Table 2). Mean number of protoplasts produced was predicted to increase by 1.857 $\times$ 10^8 protoplasts/ml for every 0.3% increase in enzyme concentration at a given incubation time, and by 0.95 $\times$ 10^8 protoplasts/ml

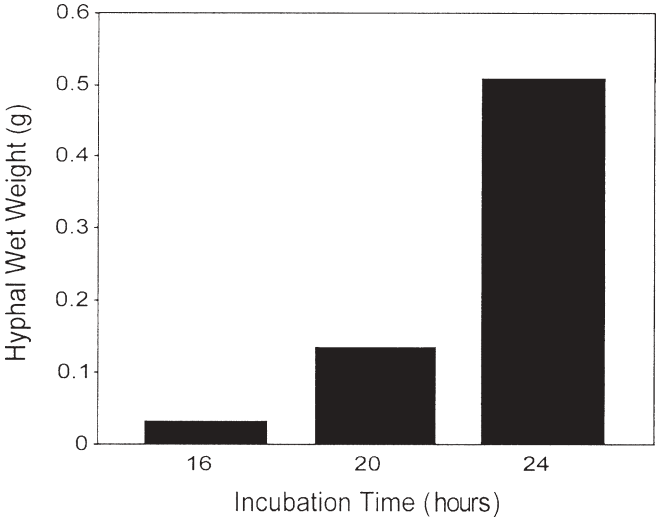

Fig. 1. Effect of incubation time on wet weight of germlings of *Ophiostoma picea* in $MgSO_4$-maleic-NaOH buffer with 1.0% lytic enzyme

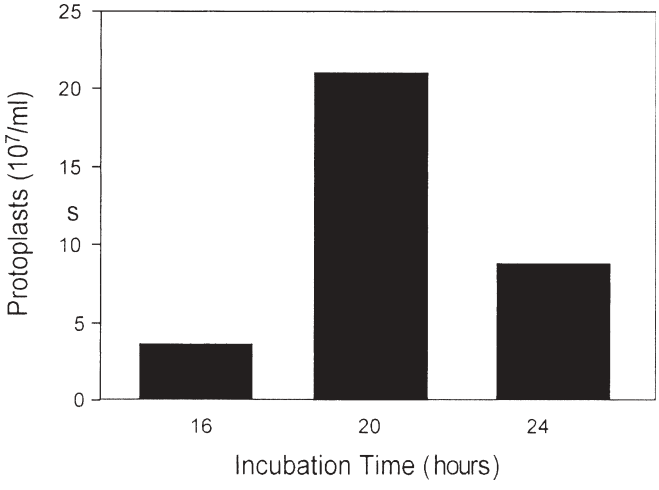

Fig. 2. Effect of incubation time on protoplast production from germlings of *Ophiostoma picea* in $MgSO_4$ -maleic-NaOH buffer with 1.0% lytic enzyme

for every 30 min of incubation at a given enzyme level. These results are not surprising because cell wall removal depends on both enzyme concentration and exposure time.[10]

Increasing enzyme concentrations and treatment times were also both associated with higher percentages of hyphal fragments, which consume enzyme, but do not contribute to protoplast production ($p < 0.0001$ and $p = 0.0019$, respectively). It is unclear why these fragments increased in frequency. Longer exposures and higher concentrations of enzyme may have increased the potential for incomplete cell wall lysis on older hyphae, which then failed to release protoplasts due to the decreased hyphal viability.

Cells that fluoresce after exposure to FITC-WGA are indicative of incomplete cell wall removal on the protoplast. In theory, the frequency of fluorescing cells should decline

Table 2. Effect of enzyme concentration and exposure period on release of protoplasts from 20-h-old germlings and subsequent regeneration of hyphae from these treatments

Enzyme concentration (%)	Incubation period (min)	Protoplasts ($\times 10^7$/ml)	Protoplasts with cell wall (%)	Hyphal fragments (%)	Regeneration (%)
0.4	30	3.32 (1.14)[a]	3.74 (0.31)	6.84 (1.98)	–[b]
	60	22.13 (0.46)	13.99 (6.73)	16.73 (6.46)	1.35 (0.18)
	120	30.75 (2.08)	10.62 (0.54)	19.79 (5.76)	0.68 (0.01)
0.7	30	29.07 (4.08)	3.75 (0.81)	15.73 (3.15)	2.17 (0.33)
	60	27.77 (12.49)	4.44 (0.46)	22.74 (8.09)	0.64 (0.13)
	120	23.37 (5.64)	14.48 (1.39)	21.84 (2.53)	1.61 (0.13)
1.0	30	17.28 (2.11)	5.22 (1.28)	29.39 (1.59)	1.07 (0.10)
	60	28.48 (2.12)	7.98 (1.29)	23.59 (1.54)	1.12 (0.27)
	120	37.63 (7.46)	8.49 (2.32)	42.36 (6.42)	0.42 (0.07)

[a] Values represent means of 3 replicates with standard deviations in parentheses
[b] There were too few protoplasts to evaluate regeneration

with increasing enzyme concentration or treatment time. Increasing enzyme concentration for a given treatment time did not significantly reduce the percentage of fluorescing cells, while increasing treatment time significantly increased the percentage of fluorescing protoplasts ($p < 0.0001$). These results imply that there was an excess of enzyme available for reaction, but that other factors, such as site access, affected cell wall removal. As a result, increasing enzyme levels had no effect on the residual cell wall. Increasing the treatment time should allow more reactions to occur and thereby improve cell wall lysis, but longer exposures will also allow lysis of cell walls on older hyphae. The cell wall polymers on these hyphae are likely to be more heavily cross-linked and therefore less likely to be completely removed.[20]

Protoplast regeneration

Although protoplast production is important, it is equally critical that the conditions used to produce protoplasts do not irreversibly damage cell functions to the point where regeneration of fungal hyphae is precluded. Excessive enzyme concentrations, prolonged treatment times, and poor buffer choices are among the factors that can hinder regeneration.

Regeneration rates of *Ophiostoma picea* were generally low, ranging from 0.4% to 2.53% for the various treatments. Increasing enzyme concentration from 0.4% to 0.7% for a given treatment time had no significant effect on regeneration, while increasing the concentration to 1.0% had a significant negative effect. Similarly, increasing treatment time to 120 min had a significant negative effect on regeneration. These results indicate that caution should be exercised to ensure that enzyme levels and exposure times adjusted to enhance protoplast release do not produce long-term impacts on cell viability and function.

Protoplasting from conidia of *O. picea* was not successful in this study. Pretreatment of conidia in 2-mercaptoethanol or dithiothreitol followed by prolonged incubation in enzyme solution may be useful, but the duration is long.[15,18] Protoplasts were easily produced from 20-h-old *O. picea* germlings after 30 min of incubation. Clearly, protoplasts can be produced from *O. picea* germlings with relative ease because this incubation time was short compared to those reported in other studies.[11,12,15,16,18] However, regeneration rates were relatively low compared to other studies.[12,16,18] Most protoplasts appeared to be intact under a light microscope, which suggests that low regeneration rates may be due to poor regeneration conditions. Further studies will be required to more completely define optimum conditions for both protoplast release and regeneration.

Conclusions

Protoplasts could be produced from 20-h-old germlings of *Ophiostoma picea* using a $MgSO_4$-maleic-NaOH buffer. Higher enzyme concentrations and treatment times increased protoplast release but reduced the ability of the protoplasts to regenerate. Exposure to 0.7% lytic enzyme for 30 min appeared to produce optimum release and regeneration for the conditions evaluated.

Acknowledgments This is paper 3564 of the Forest Research Laboratory, Oregon State University, Corvallis, Oregon, USA.

References

1. Scheffer TC (1973) Microbiological degradation and causal organisms. In: Nicholas DD (ed) Wood deterioration and its prevention by preservative treatments. Syracuse University Press, Syracuse, pp 31–106
2. Anderson RC, Hansen E, Morrell JJ (2002) Use of anti-stain chemical treatments by the western US soft wood lumber industry, 1999. Forest Prod J 52:69–71
3. Rishbeth J (1963) Stump protection against *Fomes annosus*. III. Inoculation with *Peniophora gigantea*. Ann Appl Biol 52:63–77
4. Ricard JL (1976) Biological control of decay in standing creosoted poles. J Inst Wood Sci 7:6–9
5. Bruce A, King B (1986) Biological control of decay in creosote treated distribution poles. I. Establishment of immunizing commensal fungi in poles. Mater Org 21:1–13
6. Freitag M, Morrell JJ, Bruce A (1992) Biological protection of wood: status and prospects. Biodeterior Abstr 5:1–13
7. Behrendt CJ, Blanchette RA, Farrell RL (1995) Biological control of blue-stain fungi in wood. Phytophathology 85:92–97

8. Kay SJ (1997) Biological control of sapstain in New Zealand. In: Kreber B (ed) Strategies for improving protection of logs and lumber: proceedings of symposium, November 21–22, 1997, Rotorua, New Zealand. NZ For Res Inst Bull 204:75–80
9. Morrell JJ, Gibson DG, Krahmer RL (1985) Effect of fluorescent-labeled lectins on visualization of decay fungi in wood sections. Phytopathology 75:329–332
10. Davis B (1985) Factors influencing protoplast isolation. In: Peberdy JF, Ferenczy L (eds) Fungal protoplasts: applications in biochemistry and genetics. Marcel Dekker, New York, pp 45–71
11. Gold MH, Cheng TM, Alic M (1983) Formation, fusion and regeneration of protoplasts from wild-type and auxotrophic strains of the white rot basidiomycete *Phanerochaete chrysosporium*. Appl Environ Microbiol 40:260–263
12. Kitamoto Y, Kono R, Tokimoto K, Mori N, Ichikawa Y (1984) Production of lytic enzymes against cell walls of basidiomycetes from *Trichoderma harzianum*. Trans Mycol Soc Jpn 26:69–79
13. Nutsubidze NN, Prabakaran K, Obraztsova NN, Demin VA, Su JD, Gernet MV, Elisashvili VI, Klesov AA (1990) Preparation of protoplasts from the basidiomycetes *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, and from the deuteromycetes *Trichoderma reesei* and *Trichoderma longibrachiatum*. Appl Biochem Microbiol 26:330–332
14. Trojanowski J, Huttermann A (1990) The use of protoplasts for the improvement of enzymatic activities in lignolytic fungi. Microbios Lett 43:43–48
15. Royer JC, Dewar K, Hubbes M, Horgen PA (1991) Analysis of a high frequency transformation system for *Ophiostoma ulmi*, the causal agent of Dutch elm disease. Mol Gen Genet 225:168–176
16. Rui C, Morrell JJ (1993) Production of fungal protoplasts from selected wood-degrading fungi. Wood Fiber Sci 25:61–65
17. Bartholomew K, Dos Santos G, Dumonceaux T, Valeanu L, Charles T, Archibald F (2001) Genetic transformation of *Trametes versicolor* to phleomycin resistance with the dominant selectable marker *shble*. Appl Microbiol Biotechnol 56:201–204
18. Richards WC (1994) An enzyme system to liberate spore and mycelial protoplasts from a dimorphic fungal plant pathogen *Ophiostoma ulma* (buism.) Nannf Physiol Mol Plant Pathol 44:311–319
19. Xiao Y, Wakeling RN, Singh AP (2000) Use of confocal microscopy in examining fungi in wood. Biofouling 13:231–239
20. Wessels JGH (1990) Role of cell wall architecture in fungal tip growth generation. In: Heath IB (ed) Tip growth in plant and fungal cells. Academic, San Diego, pp 1–29

The publication of this article was made possible by an Emachu Research Fund. The authors are greatful for the fund.