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Albino Strains of *Ophiostoma* Species for Biological Control of Sapstaining Fungi

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Keywords

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Summary

Albino strains of *Ophiostoma floccosum, O. piceae* and *O. pluriannulatum* were selected and screened for biological control of sapstaining fungi on New Zealand radiata pine (*Pinus radiata*). Albino strains were obtained through matings and single ascospore isolations from cultures of prevalent species in New Zealand. These strains do not synthesize the melanin-like hyphal pigments of common sapstaining fungi. Additional mating studies were also carried out to obtain isolates of *O. piceae* and *O. floccosum* that lacked pigmented fruiting structures. Several albino isolates of *O. piceae* with colorless synnemata and isolates of *O. floccosum* with little to no synnemata were obtained. Biological control potential of the albino isolates was evaluated in the laboratory by challenging them on wood chips with fungi that cause extensive sapstain in pine, *Leptographium procerum*, *Ophiostoma piliferum* and *Sphaeropsis sapinea*. Many albino isolates of *O. floccosum*, *O. piceae* and *O. pluriannulatum* were effective in stopping the challenge fungi from staining wood chips and were fast growing and colorless when grown unchallenged on wood chips. Selected albino isolates of *O. pluriannulatum* were used in two field trials in New Zealand to control sapstain. Several strains were found to significantly reduce dark sapstain as compared to the untreated control logs.

Introduction

New Zealand produces large diameter radiata pine (*Pinus radiata*) in short rotations, making it the dominant species used in its forest products industry. As with other fast growing pine species, radiata pine produces wood that consists primarily of sapwood, and is susceptible to dark discolorations due to sapstaining fungi. As a result, significant losses are incurred by the New Zealand forest products industry due to these discolorations.

Sapstain, also called blue stain, is caused by pioneer-colonizing fungi, such as *Ophiostoma*, *Ceratocystis*, *Leptographium* or *Sphaeropsis* species that utilize simple carbohydrates, fatty acids, triglycerides and other components of the sapwood (Blanchette *et al.* 1992; Farrell *et al.* 1993; Wang *et al.* 1995). The dark stain produced by these fungi is due to melanin and melanin-like compounds that are localized within the fungal hyphae (Zink and Fengel 1988; Zimmerman *et al.* 1993). As the fungus grows in wood cells, pigmented hyphae impart a discoloration to the wood (Zink and Fengel 1988; Blanchette *et al.* 1992). Sapstain fungi are not thought to compromise strength properties of wood in early stages of colonization, although discoloration decreases the value of wood used for lumber or paper production (Zabel and Morrell 1992; Seifert 1993).

Sapstain has traditionally been controlled with anti-sapstain chemicals; however, toxicity concerns and the environmental effects of many chemicals used have prompted the search for alternative methods of control. Biological control using albino strains of sapstaining fungi is a new method that can be used. Investigations using colorless strains of *Ophiostoma* species have been successful in controlling sapstain (Blanchette *et al.* 1992; Farrell *et al.* 1993; Behrendt *et al.* 1995a, b; Schmidt and Müller 1996; White-McDougall *et al.* 1998). By applying a colorless strain of *Ophiostoma* to freshly cut logs, the fungus can preferentially colonize the sapwood, thereby capturing nutrient resources and inhibiting subsequent colonization by dark staining fungi. The detrimental effects of sapstaining fungi are also important in the Canadian forest products industry. Surveys recently completed in Canada have identified *Ophiostoma* species as the most prevalent sapstaining organism (Uzunovic *et al.* 1999).

The objectives of this study were to: 1) develop colorless strains from species of *Ophiostoma* prevalent in New Zealand, 2) evaluate selected strains for their potential to control aggressive sapstaining fungi on wood, 3) obtain strains that are completely free of pigment including pigmentation in and around synnemata, and 4) to test selected albino strains in field log trials for their anti-sapstain effect.

Materials and Methods

Colorless strain selection

Strains of three common *Ophiostoma* species, *O. floccosum, O. piceae* and *O. pluriannulatum*, isolated from radiata pine in various regions of New Zealand, were used in mating studies. Isolations from stained wood were made by culturing small segments of wood on a semi-selective medium for *Ophiostoma* species amended with cycloheximide (Harrington 1981). Pure cultures were maintained on 1.5 % malt extract agar (MEA).

Matings were completed of each species to produce ascospores for single spore isolations. Tester strains of known mating types were used to determine the mating types of unknown cultures. Matings of *O. pluriannulatum* were carried out by transferring A and B strains to opposite sides of a petri plate containing 1.5 % MEA and allowing them to grow together. Perithecial formation occurred in the center of the plate where the two cultures merged. Matings of *O. floccosum* and *O. piceae* were carried out by inoculating 1.5 % (MEA) amended with several sterile pine twigs and/or wood chips. Media amended with wood was inoculated with one mating type followed by inoculation of the opposite mating type 2–3 days later. Ascospores were harvested 2–4 weeks after matings by dispersing spore droplets into sterile water. A dilute ascospore suspension was streaked onto *Ophiostoma* select medium. Spores were germinated and individually transferred to 1.5 % (MEA). Thousands of single spore isolates were evaluated for mycelium that lacked pigmentation. Selected isolates were incubated at 5 °C for 2–4 weeks. Isolates that remained colorless after this "cold treatment" were tested further in challenge experiments. Selected isolates were also used in additional mating studies to obtain a larger number of colorless strains. Many colorless mycelial isolates of *O. floccosum* and *O. piceae* continued to produce pigmented synnemata, and some isolates of *O. floccosum* produced extracellular pigment that was found around the bases of synnemata. Additional screening was done to select isolates with reduced or no pigmentation in or near synnemata stalks.

Evaluation for biological control potential

Challenge experiments were carried out to evaluate the potential of selected isolates to control sapstain produced by three fungal species isolated from stained radiata pine logs in New Zealand, *Leptographium procerum*, *Sphaeropsis sapinea* and *Ophiostoma piliferum*. These isolates cause dark staining in wood when inoculated alone. Petri plates containing three sterile pine wood chips (approx. 1.5 × 2.5 mm in size) were inoculated with 0.25 ml per chip of an albino spore suspension (approx. 6×10^6 spores/ml) and incubated for 3 days at 21 °C. Inocula for *L. procerum* and *O. piliferum* were prepared by rinsing plates of growing cultures with sterile water and the spore suspension was used to inoculate the albino treated wood chips. For *Leptographium* species and *Ophiostoma* species, each chip was inoculated with a 0.25 ml spore suspension with a concentration of approximately 6×10^6 spores/ml. Since *S. sapinea* does not produce spores in culture, inoculum was prepared by washing plates of growing cultures with sterile water and macerating the mycelium in a sterile blender to an approximate concentration of 2.5×10^5 mycelial fragments/ml. Controls for the challenge fungi were inoculated alone on wood chips. After two weeks of growth at 21 °C, stained wood chips were scored. A rating of 1 to 5 was used to evaluate the wood and mycelial coloration; 1 = white/non-stained wood, 2 = slight gray, 3 = gray, 4 = dark gray, 5 = black.

Colorless isolates were also grown on pine wood chips to monitor their growth and ability to grow pigment free. Isolates were prepared by rinsing a growing culture with sterile water, and a 5 ml spore suspension at a concentration of approximately 6×10^6 spores/ml was used to inoculate the wood chips. After a 14-day incubation period, the isolates could be visually separated using a 1–5 rating scale similar that used in the challenge study. Categories for growth were included in the 1–5 rating scale. Isolates given a rating of 1 had excellent growth and only white mycelium that completely colonized the non-stained wood chips. Isolates given a rating of 5 exhibited poor growth and/or dark staining.

Field trials with albinos on logs

Field trial 1 was established in mid November 1997 and continued until April 1998 at Kinleith Mill in the Central North Island of New Zealand. Isolates chosen for this field trial were albinos that received the highest ratings in the laboratory challenge experiments. The site for the field trial was an elevated storage site within 400 m of the forest. Six-meter logs cut from trees approximately 24 years old were obtained from the Kinleith Forest and transported to the site the day after felling. Logs were then cut into 4 pieces of 1.5 m each and randomly laid out into piles for treatment.

In Trial 1, seventeen different albino *O. pluriannulatum* strains were inoculated onto logs using a backpack sprayer at a concentration of approximately 1×10^{11} colony forming units per liter. A set of logs sprayed with only water was established as a control. Albino cultures and the water control were sprayed onto nine logs per treatment at a volume of 4 l. After 6 months in the field (November to May, the Austral summer and autumn months), the logs were assessed for coverage of sapstain on four internal surfaces of each log. All logs were sliced into 5 pieces (30 cm intervals) and each face was assessed immediately after being sprayed with water. The percentage sapstain coverage on each face was estimated by 2 groups of 2 assessors each. The assessors estimated the total amount of coverage by blue, gray and black stain at 5 % intervals on the entire transverse surface of the wood disks taken from the internal sapwood of the logs, scoring the amount of stain from 0–100 %. Statistical analysis was performed as analysis of variance and Tukey's test for comparisons of means using Minitab 12 for Microsoft Windows.

Field trial 2 was established in June 1998. The site and log parameters were the same as trial 1. The 10 logs per treatment were inoculated with the most successful albino strains from log trial 1. They included the albino *O. pluriannulatum* strains 5040, 4650 and 3410 and a control treatment with water.

The albino fungi were prepared by inoculating 10 one-liter flasks with cultures and growing in a shaking incubator at 25 °C for 2–3 days. The excess growth medium was removed by centrifugation and the fungal suspensions were resuspended in 3 l of 100 mm Tris HCl buffer at pH 7. Approximately 1×10^{12} colony forming units per albino strain were then resuspended in 50 l of water. A commercial spray unit was used for log inoculation as compared to backpack sprayers used in field trial 1. A 3 log × 3 log replicate block design was used for the trial. After 3 months the logs were assessed for stain development as in the first log trial.

Results

Mating studies

Single ascospore isolations yielded many strains that had varying degrees of desirable traits, such as reduced hyphal pigments and/or little to no synnemata production. These isolates were then incorporated into mating studies with other isolates of the same species to obtain

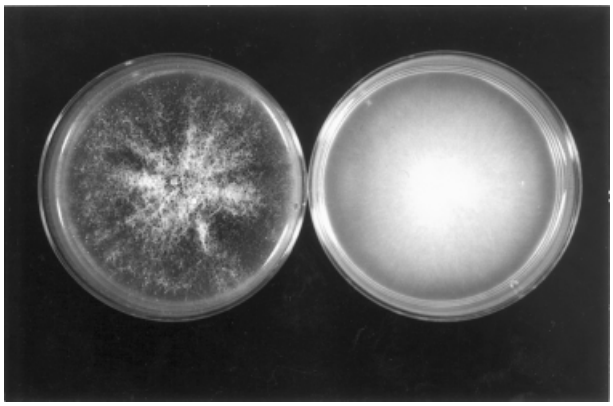

Fig. 1. Culture of a naturally occurring *Ophiostoma floccosum* (left) and an albino culture of *O. floccosum* (right).

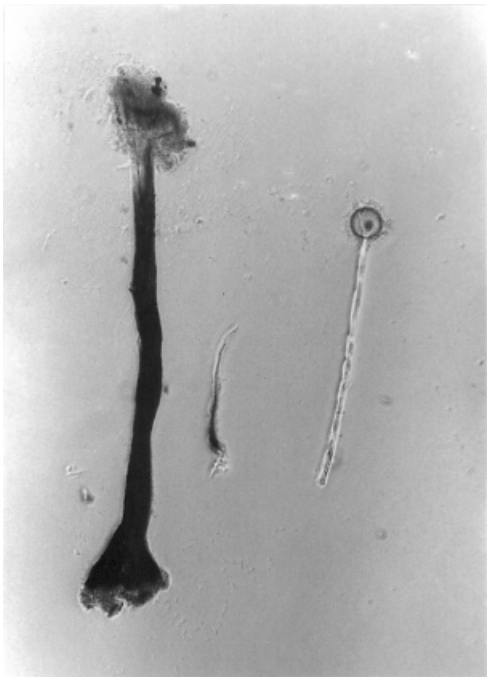

Fig. 2. Typical synemma of *Ophiostoma piceae* (left), stunted synemma of an albino isolate (center) and a hyaline synemma of an albino isolate (right).

additional colorless strains with desired characteristics. Thousands of single ascospore isolations were screened to obtain the first colorless isolates of each species. Albino strains of *O. floccosum* had colorless mycelium but retained synnemata with dark pigmented stalks. *Ophiostoma floccosum* had no pigmentation evident in albino mycelium, but a red-brown coloration remained in the synnemata. This pigmentation also extended into the medium around the base of synnemata. Continued mating studies of albino *O. floccosum* isolates produced cultures with no synnemata or with very few synnemata when grown on wood (Fig. 1). Initially selected isolates of *O. piceae* albino strains lacked pigmentation in mycelia but retained black synnemata. Additional mating experiments yielded several albino isolates that had hyaline synnemata (Fig. 2). *Ophiostoma pluriannulatum* does not produce synnemata, therefore additional matings of albino isolates were not necessary with this species. It has been shown in previous investigations using *O. piliferum* that melanin is necessary for perithecial development (Zimmerman *et al.* 1993). Therefore, matings of two albino isolates did not produce perithecia, and cannot be used in mating studies.

The "cold treatment" used to stress cultures and induce pigmentation resulted in pigment production by many isolates originally classified as colorless when grown on culturing media. These cold-stressed pigmented isolates were eliminated from further experimentation. Isolates remaining colorless after "cold treatment" screening were used in the challenge experiments.

Challenge experiments

Visual observations on wood chips inoculated with albino strains showed white mycelial growth 3 days after inoculation. Two weeks after challenging the colorless strains with the various dark sapstaining fungi, wood chips were categorized with the 1 to 5 rating scale. The results showed that not all albino isolates performed the same when challenged with dark staining fungi (Table 1). Isolates receiving a rating of 1 showed complete control of the dark staining challenge fungi, leaving the wood chips entirely white and non-stained. A rating of 5 showed failure of stain prevention and wood chips were black. Ratings of isolates between 2–4 showed some control in which wood chips were slight gray, gray or dark gray, respectively. Figure 3 shows wood chips inoculated with *O. piliferum* along with wood chips inocu-

Table 1. Percent of albino strains of *O. piceae, O. floccosum* and *O. pluriannulatum* rated for biological control potential when challenged against wild type strains of *L. procerum, O. piliferum* and *S. sapinea*. Albino strains listed as not challenged were rated for growth and ability to grow free of pigment on wood chips

Strain	Rating for biological control potential and stain production				
	1	2	3	4	5
O. piceae					
L. procerum	59	26	10	4	1
O. piliferum	82	13	5	0	0
S. sapinea	70	26	3	0	0
No challenge	63	12	25	0	0
O. floccosum					
L. procerum	90	10	0	0	0
O. piliferum	92	6	1	0	0
S. sapinea	75	21	4	0	0
No challenge	48	14	6	8	24
O. pluriannulatum					
L. procerum	8	34	43	15	0
O. piliferum	55	10	30	4	0
S. sapinea	11	16	30	43	0
No challenge	22	35	24	19	0

1 = white/non-stained wood, 2 = slight gray, 3 = gray, 4 = dark gray, 5 = black

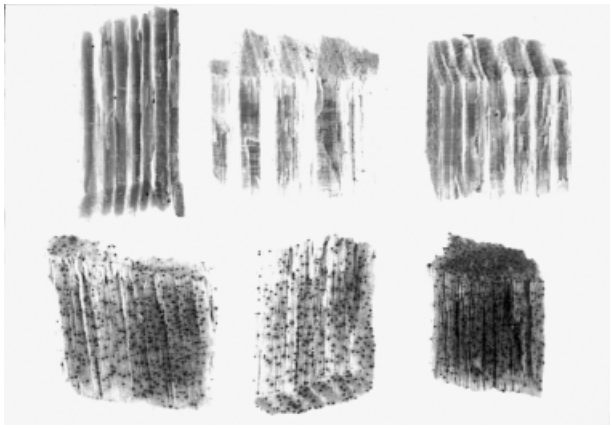

Fig. 3. Wood chips inoculated with an albino strain of *Ophiostoma piceae* followed 3 days later by a staining isolate are shown in the top row. No stain is present in these wood chips. Bottom row of darkly stained wood chips were inoculated with a staining isolate of *O. piliferum*.

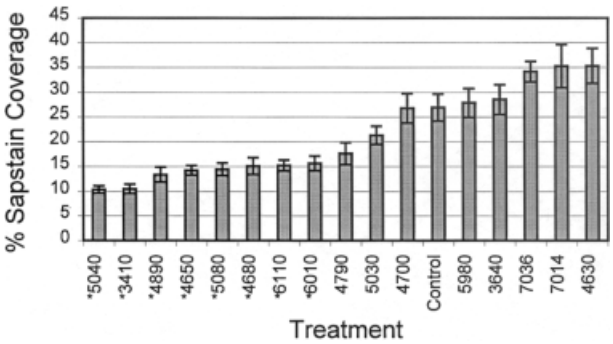

Fig. 4. Visual evaluation of sapstain on logs from field trial 1 treated with *O. pluriannulatum* albino strains and control logs receiving only water with outlying data points removed. Treatments with an * had significantly less (p=0.05) stain than the control.

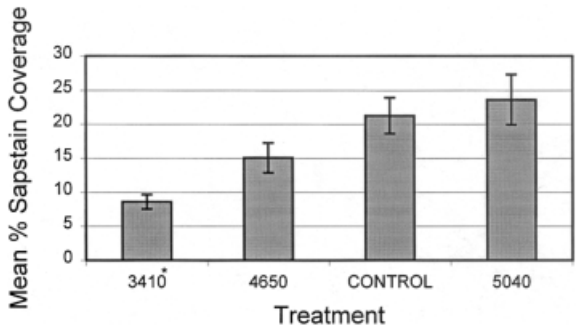

Fig. 5. Visual evaluation of sapstain in logs from field trial 2 showing the mean percentage of sapstain coverage for treatments. Treatment with an * had significantly less (p=0.05) stain than the control.

lated with a colorless *O. piceae* isolate followed by inoculation with an *O. piliferum* staining isolate. The albino strain successfully colonized the wood and prevented the stain-causing fungus from growing in the wood.

The percent of albino isolates in each rated category after challenging with 3 different dark staining fungi is shown in Table 1. *Ophiostoma floccosum* and *O. piceae* have a large percentage of isolates with a rating of 1 resulting in excellent control of the 3 challenge fungi. *Ophiostoma pluriannulatum* had the fewest isolates given a rating of 1, but many of these isolates gave excellent control. Growth of albino single ascospore cultures on wood chips, inoculated alone without challenge fungi to test growth characteristics and their capacity to remain colorless, exhibited variations within and among species (Table 1).

Field trial results

After 6 months, logs inoculated in the field with 17 different albino *Ophiostoma* strains were assessed. With an ANOVA (analysis of variance) of the data and outliers removed from the mean stain values, log treatments with eight albino *O. pluriannulatum* strains (5040, 3410, 4890, 4650, 5080, 4680, 6110 and 6010) showed significantly less stain than the control logs (Fig. 4). There was a group of albinos that were not significantly different from the control logs and a group (7036, 7014, and 4630) that were statistically more stained than the controls (Fig. 4). Since log diameter varied in this trial, the logs were assessed with regard to diameter and examined for potential confounding effects of sapstain coverage. Log diameter had no influence on the level of discoloration in this trial.

In a second log trial established in June 1998, albino *O. pluriannulatum* strains 3410, 5040 and 4650 from the first trial were again inoculated onto radiata pine logs. This trial showed that albino *O. pluriannulatum* strain 3410 had significant reduction in the amount of sapstain in comparison to control logs (Fig. 5). The other two albino *O. pluriannulatum* strains (5040 and 4650) were not statistically different from the control logs in the amount of sapstain (Fig. 5).

Discussion

These studies show that colorless *Ophiostoma* isolates can be obtained to significantly reduce stain in logs of *Pinus radiata*. Using single ascospore isolations and a series of mating studies, a large number of colorless strains were obtained from different species of *Ophiostoma*.

In the process of developing the colorless strains used in this investigation, cultures of *O. piceae* and *O. floccosum* with varying melanin production have been described. Although the mechanisms of melanin formation are not completely known, this study suggests that different genetic factors are likely to be responsible for overall pigment production in *Ophiostoma*. Pigment production in the vegetative mycelia and pigments produced in and around synnemata required different mating selections to obtain melanin-free cultures. Further studies of these mechanisms could lead to a better understanding of stain production by these fungi. Pathways for pigment formation may be similar between

O. floccosum and *O. piceae* because of their related phylogeny in the *O. piceae* complex (Harrington *et al.* 2001).

By using *in vitro* screening methods involving cold treatments and challenge experiments, isolates with optimum biological control potential were separated from other less effective cultures. The strains generated in this study are also being screened and utilized for other purposes such as biological pretreatment of wood to remove wood extractives and alleviate pitch problems during pulp and paper production (Farrell *et al.* 2000). Previous studies have shown that albino strains of *Ophiostoma* are capable of degrading wood extractives, including triglycerides, fatty and resin acids (Blanchette *et al.* 1992; Farrell *et al.* 1993, 2000; Brush *et al.* 1994; Wang *et al.* 1995). Degradation of these extractives from wood chips before they are used in the paper making process indicates these strains could have significant application in new bioprocessing technologies as depitching agents.

Melanin production in fungi is thought to be important for resistance to microbial lysis and protection from ultraviolet light and desiccation (Bloomfield and Alexander 1967; Brasier 1978). It also plays a role in perithecial development (Zimmerman *et al.* 1993). But the decreased amount or lack of melanin in the albino strains obtained in this study does not appear to inhibit the aggressiveness or growth characteristics of the fungi. The laboratory challenge experiments demonstrate that albino strains of *Ophiostoma* can be effective at preventing stain occurring from different species of sapstaining fungi. In addition to an isolate of *O. piliferum* that caused dark stain, New Zealand isolates of *L. procerum* and *S. sapinea* that also cause dark stains were inhibited. These results support previous findings (Behrendt *et al.* 1995a, b; Müller and Schmidt 1995; Blanchette *et al.* 1997) that showed some success using albino strains to control sapstain in preliminary experiments. Concerns that biological control using one organism may not sufficiently control many different species of stain fungi have been raised (Kang and Morrell 2000). Results presented here demonstrate a single albino strain can control several different genera of stain fungi that are commonly found in New Zealand timber products (Farrell *et al.* 1997).

The studies described in this paper provide important new information on the ability of laboratory selected albino strains exhibiting superior biocontrol potential to be used to reduce sapstain on radiata pine in New Zealand. This research also demonstrates that the most prevalent and aggressive *Ophiostoma* species native to a specific country can be used to obtain albino strains for biological control. This avoids the problems associated with introducing foreign strains of fungi into areas in which they are not native.

The effectiveness of controlling sapstain varied among strains tested in field trials. The second log trial was done to evaluate several isolates from the first log trial at a different time of year (June to November). Although isolates 4650 and 5040 did not perform significantly better than the control in this particular study, isolate 3410 showed significant sapstain control from the control logs. Strains 4650 and 5040 may not have performed as well as in trial 1 due to prior contamination of logs with wild type sapstaining fungi during harvest and transport. Another possibility is that these strains are not ideally suited for the environmental conditions occurring at the time of the study.

Additional field experiments are warranted to evaluate more albino strains. In this study, no attempt was made to optimize field inoculation procedures or methods to insure comprehensive coverage of the debarked logs by the albino strains. The time of inoculation by the biocontrol agent is crucial and should be done immediately after cutting (Behrendt *et al.* 1995b). Additional field trials are needed to elucidate the important environmental factors that could affect the success of the albino strains and result in more effective control. Biological control of sapstain over a 6-month period is also an exceedingly long time for the albino strains to remain efficacious. Previous investigations indicate that albino strains performed well as biocontrol agents over a shorter period in field studies (Behrendt *et al.* 1995a, b). Logs used in New Zealand field trials were completely debarked, which resulted in the entire circumference of the logs being exposed. This large area of exposed sapwood and inadequate inoculum coverage may have contributed to a reduced effectiveness of sapstain control. Improved methods of inoculation and application could provide better coverage and better adherence to the logs. When using biocontrol agents, the timber industry also may have to accept shorter periods of log storage (2–3 months instead of 6) and more rapid processing of logs treated with biocontrol fungi.

The results presented demonstrate that biological control using albino stains of *Ophiostoma* can be successful in New Zealand, but more research investigation is needed to optimize these biological control processes. A large number of albino strains of *O. piceae*, *O. pluriannulatum* and *O. floccosum* are available from this study and can be used in continued field evaluations in New Zealand to control sapstain in radiata pine logs and for other bioprocessing technologies.

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