POTENTIAL OF DAIRY PRODUCTS AND INTEGRATED SYSTEMS FOR IN-FOREST APPLICATIONS TO PROTECT *PINUS RADIATA* FROM FUNGAL DEGRADE

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ABSTRACT

Laboratory and field studies were conducted to investigate the potential of dairy products and integrated systems, containing fungi and biocides, for controlling fungal invasion of *Pinus radiata* D. Don by wood-inhabiting fungi.

In laboratory and field trials, branches and log billets of *P. radiata* were subjected to various experimental treatments prior to exposure to fungal infection. After 4 and 9 weeks of incubation, billets were visually assessed for internal discoloration. In the laboratory, satisfactory control against staining fungi was obtained with integrated systems containing *Trichoderma viride* Pers.: S.F.Gray plus sodium fluoride, *T. viride* plus sodium fluoride plus Sentry®, and a suspension of Camembert cheese rind in pasteurised milk. In the field, *T. harzianum* Rifai or *T. viride* plus sodium fluoride plus Sentry® controlled detrimental discoloration in log billets exposed to natural fungal invasion.

Keywords: discoloration; dairy products; integrated preservative systems; *Pinus radiata*.

INTRODUCTION

New Zealand is a major exporter of *Pinus radiata* logs, total exports in the year ending June 2000 being 6.081 million m^3 (NZFOA 2000). The main log markets for New Zealand are Japan and the Republic of Korea, with significant, yet potentially growing markets in the United States, China, India, and several other South-east Asian countries.

To deliver clean logs to present and future export markets, antisapstain treatments containing fungicide(s) are commonly applied in New Zealand (Wakeling 1997). In general, an antisapstain treatment provides an envelope of chemical(s) at the log's surface, and this envelope effectively acts as a barrier against invading fungi if logs are treated within 24 hours of tree felling (Eden *et al.* 1997). Immediate application of an antisapstain treatment is

required because key wood-inhabiting fungi can rapidly penetrate the surface of logs and progress to beyond the reach of a topical antisapstain treatment (Uzunovic & Webber 1998). This becomes a particular problem in areas of the log where the bark has come off, or has been peeled back during the harvesting process (Wakeling 1997). With the recent development of a mobile antisapstain product, which was designed specifically for protection of unseasoned export logs, satisfactory control of fungal pre-infections can be achieved in logs, even in summer conditions, providing the treatment is applied within 3 to 4 days after harvesting (Wakeling *et al.* 2000). In practice, however, extended ($\geq$ 4 days) time delays between tree felling and subsequent antisapstain treatment of logs are often inevitable. Such delays will be exacerbated with an increasing percentage of the future harvest being in more inhospitable, hilly country (Riddle 1997). Harvesting of logs during New Zealand summer often results in logs containing advanced fungal pre-infections, which cannot be arrested with the current mobile antisapstain treatment.

A possible approach to prevent the development of sapstain in logs that are being held for more than 4 days before application of an antisapstain treatment, may be the use of treatments which can be applied to logs immediately after harvest. However, such an approach will exclude traditional antisapstain treatments containing fungicides because the temporary nature of logging sites in forests would preclude construction of application facilities for containment to prevent environmental contamination. Therefore, benign treatments must be used in the forest with the aim of affording protection against fungal invasion for a short period (3 to 4 weeks) until an antisapstain treatment can be applied. One on-site posibility for the immediate treatment of logs may include the use of fungi or bacteria as biocontrol agents (Freitag *et al.* 1991; Morrell & Dawson-Andoh 1998; Schoeman *et al.* 1999). Biocontrol agents have been studied extensively for the protection of unseasoned sawn timber during storage and transport but less so for logs (Bernier *et al.* 1986; Croan & Highley 1992; Kreber & Morrell 1993; Behrendt *et al.* 1995a; White-McDougall *et al.* 1998; Yang 1999; Payne *et al.* 2000). Another approach for controlling fungal pre-infections may include integrated systems combining biocontrol agents with low levels of biocides (Dawson-Andoh & Morrell 1992; Behrendt *et al.* 1995b; Mankowski *et al.* 1997). In addition, natural products derived from plants or micro-organisms have received some attention for protection of unseasoned wood (Croan & Highley 1993, 1996; Hill *et al.* 1997; Ayer & Trifonov 1999). However, in the studies cited, consistent protection of unseasoned wood from sapstain has rarely been demonstrated in the field; thus a non-chemical solution remains an elusive goal in wood protection.

In this paper, we describe laboratory and field trials which were aimed at identifying possible candidates for inhibiting fungal invasion of freshly felled *P. radiata*.

MATERIAL AND METHODS

Laboratory Trials

Wood substrate

Freshly cut branch billets (250 mm long, 30–50 mm in diameter) of *P. radiata* were used. Surfaces of billets were locally damaged using a hammer and a bone scalpel to bruise and scrape off some bark to simulate the surface of a *P. radiata* log after harvesting.

Preparation of fungal additives to in-forest treatments

Candidate in-forest treatments were prepared as shown in Table 1. For *Trichoderma viride* and *T. harzianum,* spores and fragments of mycelium were scraped off a malt agar petri dish using sterile water. Each fungal suspension was decanted into 400 ml of treating solution containing fungicide(s) or sterile water (Table 1). For *Phlebiopsis gigantea* (Fr.:Fr.) Massee (synonym *Peniophora gigantea* (Fr.) Massee) strain Myc., one malt agar plate, which showed abundant mycelial growth, was blended in 400 ml of sterile water. *Gliocladium roseum* Bain (*strain* #3214*)* was prepared following the procedure described by Yang (1999). Individual malt agar plates containing the Brie fungus or the Camembert fungus, which had been isolated off the rinds of the respective cheeses, were blended with water.

TABLE 1–List of candidate in-forest treatments used on *Pinus radiata* billets.

Trial series*	Treatment†
Series 1	*Trichoderma viride* + sodium fluoride (4% NaF)
	T. viride + Sentry® (0.04% methylene bisthiocyanate (MBT))
	T. viride + 4% NaF + Sentry® (0.04% MBT)
Series 2	*T. viride*
	T. harzianum
Series 3	Ferndale Camembert cheese rind (33 g) + pasteurised milk (300 ml)
	Ferndale Brie cheese rind (31 g) + pasteurised milk (250 ml)
	Brie fungus (*Penicillium* sp. isolated from Ferndale brie)
Series 4	Milk pasteurised + homogenised
	Milk unpasteurised
	Gliocladium roseum + 4% sodium carbonate + 1% sodium bicarbonate
	Phlebiopsis gigantea
Series 5	Yoghurt (10%) in 300 ml water
	Yoghurt (10%) in 300 ml pasteurised milk
	Camembert cheese rind (16.6 g) + 284 ml water
	Camembert cheese rind (20.2 g) + 280 ml pasteurised milk
	Camembert fungi + 300 ml water

* = a control (water) treatment was included for each series
† = each treatment was made up in 400 ml sterile water unless stated otherwise

Preparation of inoculum containing sapstain fungi

Alternaria sp., *Ophiostoma piliferum* (Fr.:Fr.) Syd. and P. Syd. strain 41/91, and *Sphaeropsis sapinea* (Fr.) Dyko & Sutton *(*synonym *Diplodia pinea)* strain 2/94 were incubated on individual malt agar petri dishes until abundant mycelium had developed. A cocktail (600 ml) of inoculum, which contained a mixture of spores and hyphal fragments of the three species, was then prepared by blending together a petri dish of each species.

Treatment of billets

For each treatment, 16–24 billets were treated either by spraying, dipping, or painting, but treatment uptake was not determined. Thereafter, billets were incubated in a sterile plastic bin covered with aluminium foil for 48 hours at 25°C. The cocktail containing a mixed inoculum of sapstain fungi was then sprayed on to individual billets of a treatment set. Each

container was again covered with aluminium foil, and incubated for 4 weeks at 25°C. For controls, eight to 10 billets infected with sapstain fungi were treated with water and then incubated following the same procedure.

Assessment of billets

After 2 and 4 weeks' incubation, a subset of billets (8–12) was removed at random from each treatment set, along with at least four control billets. Ten discs, 10 mm thick, were cut at regular intervals along the length of each billet. The newly cut cross-sections were immediately rated from zero (no stain) to five (76–100% stain) according to the percentage cover of fungal stain (Table 2). However, the treatments in Series 5 were assessed after 24 hours because coloration developed and intensified up to 24 hours after cutting of the discs. A mid-range stain cover value (% MSC) was then calculated for each treatment of the five series. For example, the MSC of rating 2 with a stain cover of 6–25% was 15.5%.

TABLE 2–Rating system used to quantify fungal stain on cross-sections of discs.

Rating	Stain cover (%)	Mid-range stain cover (%)
0	0 (no growth)	0
1	1 – 5	3
2	6 – 25	15.5
3	26 – 50	38
4	51 – 75	63
5	76 – 100	88

Log Billet Field Trial

Substrate used

Pruned logs (4 m long, 150–300 mm in diameter) of freshly felled *P. radiata* were transported to a local sawmill for debarking, cut into 0.5-m-long sections, and then labelled. Thereafter, billets were transported to the Forest Research Institute and stacked at random in one pile prior to treatment.

Treatment of log billets

Thirty litres of each candidate treatment were freshly prepared just prior to the dipping of log billets (Table 3). Uptake of treatment solution was not determined. For each treatment, 10 log billets were selected at random, and each billet was partly immersed and then rotated in the treatment solution for 10 seconds to ensure complete wetting of the wood surface. After treatment, the cross-cut ends of the 10 billets within a treatment set were immediately sealed with bitumen to prevent excessive moisture loss through the ends, and then stack-piled inside a pole barn for 48 hours. After 48 hours, all logs were moved outside on to CCA-treated bearers and covered with black weed mat to provide some protection against sun, wind, and rain. The outside storage area was a concrete floor, which was sheltered from the wind by three 1.5-m-high stone walls, but no roof.

Assessment of log billets

The billets were assessed after 4 and 9 weeks for development of fungal degrade. At each assessment, five billets of each treatment set were selected at random. Each billet was then

TABLE 3–List of treatments used for log billets.

Treatment	Abbreviation
Ferndale Camembert cheese rind (500g) blended in 3 litres of unpasteurised nilk	Camembert
Unpasteurised milk (3 litres)	Milk
Gliocladium roseum (6.73 x10^5 propagules) + sodium carbonate + sodium bicarbonate	*G. roseum*
Trichoderma viride (1.2 x 10^7 propagules) + 4% sodium fluoride + Sentry® (0.04% MBT)	*T. viride* + NaF + Sentry
T. viride + 5% boron + Sentry® (0.04% MBT)	*T. viride* + B + Sentry
T. harzianum (3.5 x 10^7 propagules) + 4% sodium fluoride + Sentry® (0.04% MBT)	*T. harz.* + NaF + Sentry
Untreated	Control

cross-cut into five discs (120 mm thick) using a chainsaw. The four newly-cut faces of the discs were visually inspected, and the extent of discoloration was assessed using the rating system described in Table 2. At the first assessment, a rating was given for discoloration, but it was separated into bluestain and discoloration caused by decay fungi at the second assessment. For each treatment, the ratings from the surface of 20 discs were used to determine an average mid-range stain cover (%MSC) for bluestain and decay, along with the standard deviation.

RESULTS AND DISCUSSION

Laboratory Trials

The series of laboratory trials suggested several promising in-forest treatments to provide protection against staining fungi (Table 4). In Series 1, billets treated with an integrated control system containing *T. viride*, sodium fluoride, and Sentry® were entirely free of fungal degrade after 4 weeks' incubation. Application of *T. viride* plus either sodium fluoride or Sentry® yielded 7% MSC and 18% MSC, respectively, and the latter was not considered a satisfactory level of protection. The results suggest that for effective control against the three staining fungi, all components (*T. viride*, sodium fluoride, Sentry®) of the integrated system were required in a single treatment, indicating a possible complementary mode of action.

Camembert cheese rind suspended in pasteurised milk gave very good control of fungal degrade, with an MSC of 4% (Table 4, Series 3). Although in Series 5 trials this combination was not so effective (MSC 18%), it gave greater control than Camembert cheese rind in water (MSC 29%) or a suspension of the Camembert cheese rind fungus in water (MSC 39%). This indicated that the specific combination of Camembert cheese rind and pasteurised milk was required to inhibit fungal degrade, pointing to a possible complementary or synergistic mode of action of these two dairy products. Likewise in Series 5, pasteurised milk enhanced the bioactivity of yoghurt (MSC 20%) compared to a suspension of yoghurt in water (MSC 39%). Interestingly, billets treated with unpasteurised milk gave 11% MSC after 4 weeks' incubation, but pasteurised milk did not control or reduce fungal degrade when compared to

TABLE 4–Average mid-range surface coverage (MSC%) on treated *P. radiata* billets after 4 weeks of incubation

Trial series*	Treatment†	MSC (%)
Series 1	Water	36
	Trichoderma viride + NaF	7
	T. viride + Sentry®	18
	T. viride + NaF + Sentry®	1
Series 2	Water	25
	T. viride	27
	T. harzianum	13
	Egg white	28
Series 3	Water	17
	Camembert cheese rind (11%) + pasteurised milk	4
	Brie cheese rind (12.4%)+ pasteurised milk	12
	Penicillium sp. isolated from brie	10
Series 4	Water	21
	Milk pasteurised + homogenised	21
	Milk unpasteurised	11
	Gliocladium roseum + 4% sodium carbonate + 1% sodium bicarbonate	19
	Phlebiopsis gigantea	45
Series 5	Water	62
	Yoghurt (10%) in water	39
	Yoghurt (10%) in pasteurised milk	20
	Camembert rind (5.8%) in water	29
	Camembert rind (7.2%) in pasteurised milk	18
	Camembert fungi + water	39

* = a control (water) treatment was included for each series
† = each treatment was made up in 400 ml sterile water unless stated otherwise

controls. This confirmed a previous observation suggesting that unpasteurised milk has some fungicidal properties (Bettiol 1999). The results of this study indicate that manufacturing of unpasteurised milk into pasteurised milk, which includes the step of milk homogenisation, causes loss of fungicidal activity.

Brie cheese rind plus pasteurised milk, and *Penicillium* sp. isolated from Brie cheese rind had MSC of 12% and 10% respectively, suggesting some potential for controlling fungal degrade. However, egg white, *G. roseum,* and *Phlebiopsis gigantea* did not reduce fungal degrade under the conditions of the laboratory trial. Strains of *P. gigantea* and *G. roseum* have been reported to control sapstain on North American softwood logs (Behrendt & Blanchette 1997; Yang 1999), but failure to grow prolifically on *Pinus radiata* billets may have negatively affected their activity.

While the present laboratory trials focused primarily on identifying trends in the ability of candidate treatments to afford protection of billets against staining fungi, some general statements on treatment variability cannot be omitted. Firstly, gradients in the level of protection afforded by a treatment were observed within individual billets. In general, the outer one to two discs (20 mm deep) cut from both ends of a billet showed higher levels of staining than the centre portion. This highlights the difficulty of controlling fungal invasion, which develops through the cross-cut ends of billets, and corroborates our general experience

from the field. Secondly, variation occurred between billets of a treatment. This is also a common phenomenon in the field. The exact nature of the variability between billets is unclear and warrants further research.

Log Billet Field Trial

After 4 weeks of storage, the majority of billets showed considerable fungal growth on the wood surface, except for the treatments with *T. viride* and *T. harzianum*. In general, the groups of fungi seen on the surface of billets were moulds, and staining and decay fungi. Further visual examination of the log surface indicated that the fungi belonged to *Trichoderma* sp. and *Phlebopsis gigantea*, a white-rot fungus common on *Pinus radiata* (Keirle 1980). However, only minor discoloration was noted when log billets were cross-cut. The average degree of bluestain did not exceed 5% MSC for any treatment after 4 weeks, but this cannot be considered to indicate the absence of fungal infection. To determine the extent of fungal colonisation, sampling of wood chips from different depths below the log surface and growing on nutrient media is required, or use of molecular probes that detect specific groups of fungi. The low degree of discoloration observed in the 4-week-old billets agrees with our experience from previous log billet trials where fungal discoloration is often delayed until 6–8 weeks of log storage.

Application of an integrated system containing *T. harzianum* to log billets provided superior protection against development of discoloration (Fig. 1). After 9 weeks of storage,

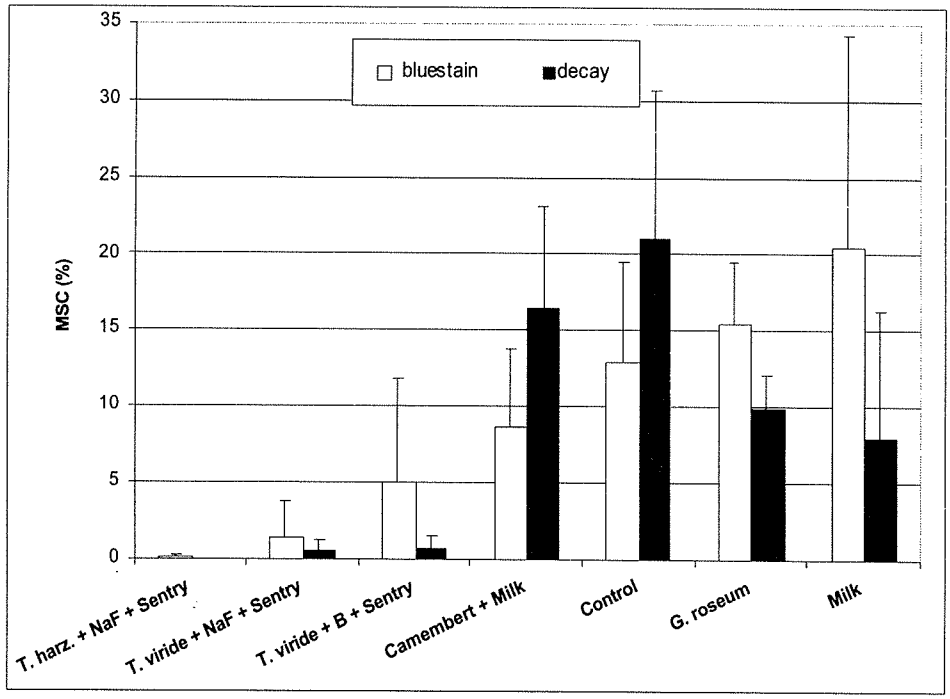

FIG. 1–Average discoloration (%) determined for treated log billets stored for 9 weeks (bars refer to standard deviation).

log billets treated with *T. harzianum* plus sodium fluoride plus Sentry® were virtually free of discoloration. *Trichoderma viride* plus sodium fluoride plus Sentry® was also effective and only minor discoloration (mean MSC ≤ 5%) was observed. Data from other field studies showed that 0.125% weight/volume (%w/w) of Sentry® does not control fungal infection of *P. radiata* (Wakeling *et al.* 2000). In a related laboratory study, Singh *et al.* (2001) showed that only growth of *S. sapinea*, but not *Leptographium procerum* (Kendrick) M.K.Wingfield and *Ophiostoma flocossum* Mathiesen, was inhibited on *P. radiata* treated with 0.04% w/v methylene bisthiocyanate. These observations provide some evidence that control of discoloration on *P. radiata* billets was not due to the fungicides alone but was the result of a complementary mode of action of the integrated system.

Although the treatments just described satisfactorily controlled invasion by staining and decay fungi, other treatments were less successful in preventing both groups of organisms. For example, Camembert cheese rind plus non-pasteurised milk gave reasonable protection against stain, but not decay fungi. The apparent weakness of the Camembert cheese rind treatment was not identified in the laboratory trial, which focused exclusively on control of staining fungi.

CONCLUSIONS

An integrated system containing *T. harzianum* plus sodium fluoride and Sentry® gave satisfactory control against fungal discoloration in sapwood of unseasoned *P. radiata*. Further work is needed to determine the depth and speed of *T. harzianum* penetration into the sub-surface of logs during storage. In addition, the minimum strength of sodium fluoride and Sentry® required to effect satisfactory control of discoloration warrants additional research to reduce the potential impact on the environment. There is also scope to use other environmentally benign compounds that match or possibly augment the performance of the integrated system containing *T. harzianum* on *P. radiata* logs.

Treatments with selected dairy products greatly reduced fungal degrade on *P. radiata* sapwood, pointing to a new approach for protecting unseasoned wood, which may warrant future research.

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