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Joanne M. Thwaites · Roberta L. Farrell · Kunio Hata
Peter Carter · Marco Lausberg

Sapstain fungi on *Pinus radiata* logs – from New Zealand Forest to export in Japan

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Abstract Supply of clear, unstained logs from an export country is important economically as well as in reducing the biosecurity risk to an importing country such as Japan. Although conditions found within the holds of ships containing logs are thought to be ideal for rapid colonization of sapstain fungi, no research has been conducted. Research-focussed log export trials were designed to determine the extent of sapstain colonisation at specific points in the processing of logs from harvesting to arrival at the export destination. Two trials were established, in the austral summer and winter, in which mature *Pinus radiata* logs were harvested in New Zealand and shipped to export ports in Japan. Data loggers placed above and below deck of the ships recorded microclimatic conditions. Nine species of sapstain fungi were isolated from logs during the summer trial; the most common species isolated were *Ophiostoma floccosum*, *Ophiostoma querci*, and *Ophiostoma setosum*. In contrast, ten sapstain species were detected during the winter trial, with *Sphaeropsis sapinea*, *O. querci*, *O. floccosum*, and *O. setosum* most commonly isolated. This research was the first successful attempt at measuring visual sapstain development and isolating sapstain fungi from the time of harvesting to arrival at an export destination.

Key words Sapstain · *Sphaeropsis sapinea* · *Ophiostoma* species · New Zealand *Pinus radiata* · Japan

J.M. Thwaites · R.L. Farrell (✉)
Department of Biological Sciences, University of Waikato, Private Bag 3105, Hamilton 2001, New Zealand
Tel. +64-7-838-4903; Fax +64-7-838-4967
e-mail: r.farrell@waikato.ac.nz

K. Hata
Denken Inc, Tokyo 100-0014, Japan

P. Carter · M. Lausberg
Fibre-gen, Carter Holt Harvey, Auckland 1015, New Zealand

Introduction

New Zealand is one of the world's largest exporters of softwood logs. Exports of forest products provide 4% of New Zealand's gross domestic product (GDP) with the major markets for logs including Japan, Korea, China, and India. The prevention of sapstain is crucial for these high value products. The shipment of the primary tree species in New Zealand, *Pinus radiata* (D. Don), from New Zealand to these export destinations requires lengthy transport times and crossing of the equator where moist warm conditions that can promote fungal growth are often encountered.

As global movement of wood and wood products increases, so does the threat of the introduction of nonindigenous fungal species. Wingfield et al.[1] described that little is known about the intercontinental spread of pathogens that infect solid wood, especially the sapstain fungus *Sphaeropsis sapinea* (Fries:Fries) Dyko and Sutton which is now widespread through exotic pine plantations. *Ophiostoma* species have also spread from native pine-growing areas to exotic plantations.[1] As an exporter of forest products, New Zealand has a responsibility to ensure that its exports do not constitute a biosecurity risk to the importing country.[2]

Sapstain is the cosmetic discoloration of wood caused by the pigmented hyphae of fungi belonging to several taxonomic groups. The stain may be superficial or penetrate deeply into the sapwood causing a stain that is blue, grey, or black. This discolouration does not affect the integrity of the wood but affects domestic and export earnings for the forest industries. *Pinus radiata* is highly susceptible to colonisation by sapstain fungi with an estimated annual loss in revenue of NZ$100 million per year.[3]

Historically in New Zealand, the major sapstain problems of *P. radiata* were associated with *S. sapinea*.[4,5] Other minor sapstain species were also found including various *Ophiostoma* species.[5,6] A more comprehensive survey of sapstain fungi in New Zealand was conducted from 1996 until 1998.[7] The sapstain fungi isolated and identified in-

cluded *S. sapinea* and 13 members of the Ophiostomataceae family.[7]

Currently, chemical control of sapstain is the most commonly used method to protect high value logs and sawn lumber.[3] Antisapstain chemicals inhibit fungal growth by the formation of a thin barrier that prevents the germination of fungal spores on the wood substrate.[8] These chemicals are applied to the surface of lumber by dipping into tanks or by spraying. The most common antisapstain products currently used in New Zealand include the chemicals copper-8-quinolate or didecyldimethyl ammonium chloride (DDAC) as the active antifungal ingredient.[9]

The aim of this present study was to verify, in a controlled and measured environment, the ecological patterns of sapstain colonisation at specific points in the processing of logs from harvesting to an export destination. The research-focussed log export trials also compared the effect of shipment in two different seasons (austral summer and winter) and monitored environmental conditions during storage and shipment of the logs. A comparison of the effects of antisapstain treatment on sapstain colonisation was also performed.

Material and methods

Two field trials were established in significantly different climatic conditions (austral summer and austral winter) of generally the same design, in which mature harvested logs were treated with an antisapstain chemical 4 days after harvesting and shipped to Japan.

Trial 1 – summer 2001

The first research-focussed log export trial (export trial 1) was established on 7th February, 2001 at a storage yard in Carter Holt Harvey's Kinleith Forest, Central North Island, New Zealand. Six-meter logs cut from trees approximately 24 years old, with an average diameter of 27 cm, were obtained from Kinleith Forest. There was a total of 47 logs in the field trial; 23 logs were untreated controls and 24 logs were treated with antisapstain chemical 4 days after harvesting. For the untreated control, logs were not debarked because this practice represented the normal export of logs to Japan at the time of this trial and the logs had no antisapstain treatment. The antisapstain-treated logs were debarked 4 days after harvesting and sprayed with the benchmark antisapstain product Cutrol 375, (Fernz Timber Protection, Auckland, New Zealand) containing the active ingredient copper-8-quinolate at a final concentration of 0.36% active ingredient. The concentration level of this product was determined by the industry and was justified on the basis of cost and efficacy. A pink dye was also added to ensure good coverage of the antisapstain treatment. The logs were stored in the Kinleith trial area until transportation to the Port of Tauranga, New Zealand, and subsequent shipment to Japan aboard the transport ship MV Nin. The export trial 1 logs were off-loaded at the Port of Akita, in northern Japan in the second week of April 2001.

Table 1. Visual stain categories used in export trials to estimate the amount of sapstain coverage on sample discs

Stain category	Description
0	No stain (0%)
1	Minimally stained (1%–10%)
2	Mildly stained (11%–20%)
3	Moderately stained (21%–50%)
4	Heavily stained (51%–80%)
5	Severely stained (81%–100%)

The logs were assessed for visual sapstain appearance and sampled for fungal isolations at three time points: immediately before chemical treatment, at 5 weeks after treatment just prior to transportation to the port for loading aboard the ship, and 10 weeks after treatment upon arrival at the Port of Akita, Japan. All logs were sampled with a chain saw as follows:

1. A 0.5-m section was sliced from both ends of the log and discarded.
2. A 50-mm sample disc was then sliced from both ends of all logs.
3. Each sample disc was wetted with water and assessed for visual sapstain coverage by two independent assessors estimating the amount of visual sapstain on the face of each sample disc using the scale in Table 1. Only shades of blue, grey, or black stain were used in the estimation of stain.
4. Five random pieces, approximately $10 \times 5 \times 5$ cm were taken from each disc, placed in sterile paper bags and returned to the laboratory at the University of Waikato in order to identify fungal infection. Samples from Japan were stored for 2 days before isolations were made; otherwise all samples were processed within 24 hours.

Statistical analysis of the score of visual stain on the sample discs was performed using the program Minitab Version 12. Individual logs from each treatment were considered replicates. Differences with respect to severity of stain between treatments and time of sampling were investigated using analysis of variance (ANOVA). Analysis was conducted using Tukey's pairwise comparisons to determine the nature of the differences detected by ANOVA.

Fungal isolation

Sapstain fungal infection was determined from the five randomly sampled pieces taken from each sample disc in the laboratory. The pieces were surface sterilized by soaking in 5% hypochlorite solution for approximately 2 minutes and rinsed twice in sterile water. The samples were then slivered with a sterile scalpel, and the slivers were placed onto two selective media. The first medium consisted of yeast malt agar (0.2% yeast extract, 2% malt extract, 2% bacteriological agar) supplemented with 200 μg/ml of chloramphenicol

and 100μg/ml of streptomycin sulfate to suppress bacterial growth. The second medium selective for *Ophiostoma* species was slightly modified from that used by Harrington,[10] and consisted of yeast malt agar (0.2% yeast extract, 2% malt extract, 2% bacteriological agar) supplemented with 200μg/ml of chloramphenicol, 100μg/ml of streptomycin sulfate, and 400μg/ml of cycloheximide.

The selective media plates with wood slivers were incubated for up to 30 days at 25°C in a darkened chamber. All potential sapstain fungi were aseptically transferred onto malt extract agar plates (1.5% malt extract and 2% agar). Cultures of *Sphaeropsis sapinea* were placed on yeast malt agar containing sterile *Pinus radiata* needles under ultraviolet light at ambient temperatures for 2 weeks. The pine needles induce the formation of pycnidia by *S. sapinea*. Sterile water was added to the plates containing the inoculated needles and after 10 min the needles were examined microscopically for the presence of pycnidia and subsequent spore release. Cultures of Ophiostomataceae were identified on the basis of morphological and physiological characters into putative species. Mating capabilities with tester strains and molecular characterisation, particularly DNA sequence of the internal transcribed spacer regions of ribosomal DNA, were used to confirm species identification.[11]

At each time point, the presence or absence of individual species on the selective media was recorded. The occurrence of a species in a sample was scored as a single record regardless of the number of colonies developing on the media. The percentage of isolations for each species, at each time point and for each treatment, was calculated as the number of positive isolations of a species divided by total isolation attempts multiplied by 100.

Trial 2 – winter 2001

The second research-focussed log export trial (export trial 2) was established on the 28th of August, 2001 at the same location as export trial 1 with logs obtained from Carter Holt Harvey's Kinleith Forest. *Pinus radiata* trees, from trees approximately 24 years old, with an average diameter of 27 cm, were harvested, cut, and treated as previously described with the exception that the control logs were debarked due to concerns in the first trial of potential beetle infestation of the bark. The logs were stored in the Carter Holt Harvey log storage area in Kinleith until transportation to the Port of Tauranga, New Zealand, and shipment to Hamada, Japan, aboard the MV Rubin Forest.

Logs were sampled prior to antisapstain treatment, and after 8 weeks of storage in the log storage area just prior to transportation to the port for loading aboard the ship, and at 15 weeks on arrival at the Port of Hamada, Japan. Logs were assessed for sapstain discoloration and sampled for fungal infection using the methods described for the first export trial.

Weather information, both mean daily rainfall and temperature, for the trial periods at Kinleith were provided by a weather station within the area. Two climatic data recorders (Hobo H8 Pro RH/Temperature loggers) accompanied the logs on both ships for export trials 1 and 2, with one data logger placed above deck and one placed below deck. Measurements of temperature and relative humidity were recorded.

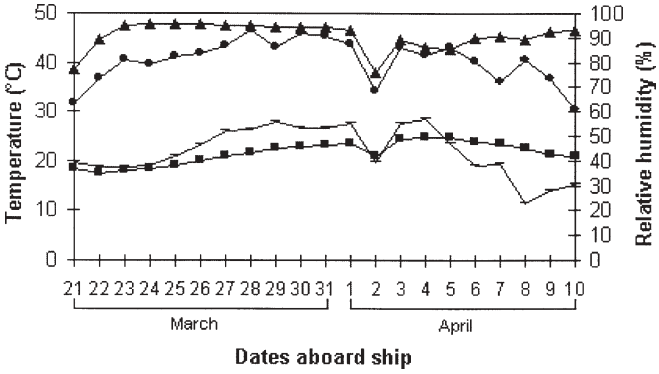

Fig. 1. Mean daily temperature and relative humidity above and below deck of the ship MV Nin for the period of transportation to Japan during export trial 1. *Minus signs*, temperature below deck; *squares*, temperature above deck; *circles*, relative humidity below deck; *triangles*, relative humidity above deck

Results

Climatic information for both export trials for the dates when logs were stored at Kinleith was measured. Export trial 1 logs were stored in New Zealand at an average temperature of 18°C with a range of 13°–22°C, for the period of harvesting and holding of the logs preshipment at Kinleith. The average rainfall for this period was 7 mm/day. The weather conditions for the Kinleith area for the winter trial showed significantly lower temperatures and rainfall than the summer trial, with an average temperature of 11°C and average daily rainfall of 3 mm for the period from harvesting to shipment of the export logs.

Two data recorders, one above deck and one below deck, measured the average daily temperature and relative humidity for the duration of the voyage to Japan in both trials (Figs. 1 and 2). For export trial 1, the logs were aboard the ship from the 21st March to 10th April, 2001. The temperatures ranged from 11° to 28°C below deck and from 17° to 25°C above deck. The relative humidity ranged from 61% to 95% below deck and from 75% to 96% above deck.

For export trial 2, the logs were aboard the ship from the 27th October to 28th November, 2001. In contrast, the temperatures were slightly lower for export trial 2 than export trial 1. The temperature ranged from 10° to 29°C above deck and from 13° to 22°C below deck. Relative humidity was approximately the same for the two trials. Relative humidity for the shipment period of export trial 2 ranged between 79% and 99% below deck and between 56% and 92% above deck.

Table 2. The total number of logs grouped according to the values of stain severity for different sample times

Stain severity	Before antisapstain treatment				Before shipment				Japan			
	Trial 1		Trial 2		Trial 1		Trial 2		Trial 1		Trial 2	
	Control	Anti-sapstain treated	Control	Anti-sapstain treated	Control	Anti-sapstain treated	Control	Anti-sapstain treated	Control	Anti-sapstain treated	Control	Anti-sapstain treated
No stain (0)	23	24	23	23	0	2	4	18	0	0	0	14
Minimally stained (1)	0	0	0	0	12	19	18	5	2	13	6	8
Mildly stained (2)	0	0	0	0	7	3	1	0	11	8	8	1
Moderately stained (3)	0	0	0	0	2	0	0	0	7	3	9	0
Heavily stained (4)	0	0	0	0	2	0	0	0	3	0	0	0
Severely stained (5)	0	0	0	0	0	0	0	0	0	0	0	0
Average severity of stain	0	0	0	0	1.7	1	0.9	0.2	2.5	1.6	2.1	0.4
Total number of logs	23	24	23	23	23	24	23	23	23	24	23	23

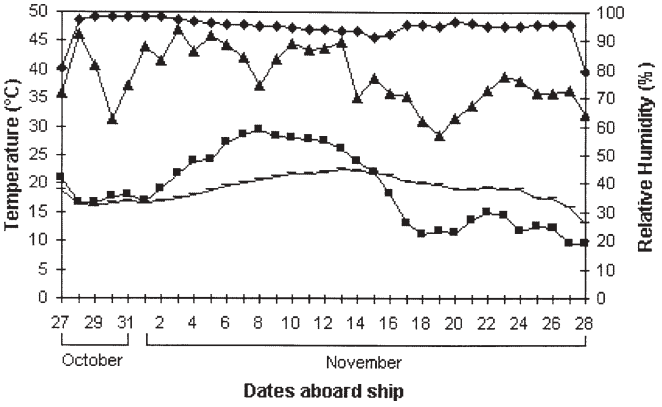

Fig. 2. Mean daily temperature and relative humidity above and below deck of the ship MV Rubin Forest for the period of transportation during export trial 2

Visual stain data

Visual stain data for both trials according to the sampling periods are provided in Table 2. No logs were stained, controls nor treated, after harvesting and prior to the chemical treatment for both trials. Before shipment to Japan, the logs from export trial 1 were more stained than the treated logs from export trial 2. Staining levels before shipment ranged from minimal (0%–10%) to heavily stained (51%–80%) in summer; there was no stain or minimal stain on the logs in winter. The shipment arriving in Japan with logs that were harvested in the austral summer had more stained logs and more sever staining than logs harvested in winter.

There was a statistical difference in the average severity of stain between the control logs with no antisapstain treatment and the logs treated with antisapstain 4 days after harvesting for trial 1 ($F = 23.4$, $P = 0.000$) and trial 2 ($F = 59.5$, $P = 0.000$). The control logs had a higher average stain severity before shipment and at the port in Japan than the antisapstain-treated logs for both export trials.

There was also a statistical difference in the average stain severity between the different sampling time points for trial 1 ($F = 118.2$, $P = 0.000$) and trial 2 ($F = 53.7$, $P = 0.000$). It should be noted for these research-focussed log export trials, the majority of logs received in Japan fell in the groups “no stain”, “minimally stained,” and “mildly stained.”

Fungal data analysis

From the 1410 samples taken, a total of 611 individual isolates of sapstain fungi were found in export trial 1. The most commonly isolated fungi, using both classical mycological techniques and molecular DNA probe techniques, in the summer trial were *Ophiostoma floccosum*, *Ophiostoma querci*, and *Ophiostoma setosum*. Six other sapstain fungal species were also isolated and identified but at lesser frequency (Table 3). A number of *Ophiostoma* species grew on the selective media but were unable to be obtained in pure culture for identification. These species were identified

Table 3. Overall frequency of individual sapstain species isolated from export trials 1 and 2

Sapstain species	Trial 1	Trial 2
Leptographium procerum (Kendrick) Wingfield	35	2
Ophiostoma sp.	86	7
Ophiostoma floccosum Mathiesen	159	23
Ophiostoma huntii (Robinson-Jeffrey) de Hoog and Scheffer	46	4
Ophiostoma ips (Rumbold) Nannfeldt	10	Not identified
Ophiostoma piceae (Münch) H. and P. Sydow	25	12
Ophiostoma piliferum (Fries) H. and P. Sydow	Not identified	2
Ophiostoma pluriannulatum (Hedgcock) H. and P. Sydow	Not identified	1
Ophiostoma querci (Georgevitch) Nannfeldt	158	78
Ophiostoma setosum Uzonovic, Seifert, Kim and Breuil	85	23
Sphaeropsis sapinea (Fries:Fries) Dyko and Sutton	7	112
Total independent sapstain isolates	611	264
Total pieces of wood sampled	1410	1840

as *Ophiostoma* species due to morphological features seen on the selective media as well as the ability to grow in the presence of cycloheximide, and are represented in Table 3 collectively called *Ophiostoma* species.

In comparison, export trial 2 established in winter 2001 had a total of 264 individual isolates sapstain fungi from 1840 samples taken. The most common sapstain fungi identified by classical and molecular DNA techniques were *Sphaeropsis sapinea*, *O. querci*, *O. floccosum*, and *O. setosum* (Table 3). In both trials sapstain fungi were isolated and identified from both stained and unstained pieces of wood.

Other wood inhabiting fungi grew on the selective media, including *Alternaria* sp., *Cladosporium* species, *Epicoccum* species, *Fusarium* species, *Mucor* species, *Penicillium* species, *Pestalotia* species, and *Trichoderma* species. These are surface-inhabiting fungi, which typically are planed off and removed from wood, were not quantified nor characterised to species level because the scope of this research was to identify the penetrating sapstain species.

The proportions of logs with *S. sapinea* and the *Ophiostoma* species, for the varying sampling periods and different treatments, are shown in Table 4. The effect of season was evident in the amount of sapstain fungi isolated when comparing export trial 1 (summer) and export trial 2 (winter). A total of 32% of samples taken prior to antisapstain treatment on the control logs for the summer trial contained *Ophiostoma* species compared with only 0.4% for the winter trial. Thus, one conclusion with regard to the effect of season on the development of sapstain was that summer harvesting has the potential for significantly more sapstain to be present, which must be aggressively treated to stop its appearance and downgrade of logs. *Ophiostoma* species were isolated in higher amounts than *S. sapinea* in both export trial 1 and export trial 2. The control logs with no antisapstain treatment had more sapstain fungi than the antisapstain chemical-treated logs, which establishes the logical conclusion that antisapstain treatment prevents the presence of sapstain fungi in exported logs.

Isolations of the *Ophiostoma* species, increased with each sampling time, from harvesting to the export destination, demonstrating that even with antisapstain chemical treatment the logs contain the fungal species that proliferate with time. In conclusion, from export trial 1, nine species of sapstain fungi were isolated, the most common being *O. floccosum*, *O. querci*, and *O. setosum*. In contrast, ten sapstain species were isolated from export trial 2 and the most common were *S. sapinea*, *O. querci*, *O. floccosum*, and *O. setosum*.

Discussion

This is the first report of sapstain development and sapstain fungal colonisation on logs from harvesting to an export destination. Farrell et al.[7] studied the sapstain fungi of New Zealand and identified *Sphaeropsis sapinea* and 13 *Ophiostoma* species as the major sapstain organisms. The fungi identified in the export trials described in this report were all species that had been previously isolated in the survey of Farrell et al. [7]

Sphaeropsis sapinea was described in the literature as the cause of the majority of sapstain problems in New Zealand.[4,5,7] The results of both export trials in this study demonstrated isolation of only a few cultures of *S. sapinea* and were, at first, a little surprising. However, the spores of *S. sapinea* are associated with cones and needles that are found more commonly on the forest floor.[12] The inoculum density of *S. sapinea* is presumably higher therefore in the forest environment. Because the logs were stored before shipment in a tidy processing yard with no branches, cones, or needles present, the incidence of infection by spores of *S. sapinea* was probably low. The reduced incidence of *S. sapinea* in logs exported to Japan is a significant finding because it demonstrates a reduction of a pathogen being exported from New Zealand. This finding also shows the importance of the export trials as reported here for determining the microorganisms being exported on logs, rather than relying on results from logs remaining in the export country.

More *Ophiostoma* species were isolated compared to *S. sapinea* in both trials. The sticky spores on the top of synemmatal and perithelial stalks of *Ophiostoma* species were shown to be disseminated by wind and insect vectors, and are commonly found on logs and timber.[13] The insect vectors, particularly the bark beetles (commonly *Hylastes ater* in New Zealand), are found in both logs and stumps.[14] Insects and wind increase the spread of *Ophiostoma* species from logs at mills, processing plants, and ports, and therefore, more inoculum potential for *Ophiostoma* species could occur at these sites.

Other dematiaceous hyphomycetes, such as *Cladosporium* species, *Alternaria* species, and *Epicoccum* species,

Table 4. Percentage of *Sphaeropsis sapinea* and total *Ophiostoma* species isolated according to each treatment and each sampling time for export trials 1 and 2

	Before antisapstain treatment				Before shipment				Japan			
	Trial 1		Trial 2		Trial 1		Trial 2		Trial 1		Trial 2	
	Control	Anti-sapstain treated	Control	Anti-sapstain treated	Control	Anti-sapstain treated	Control	Anti-sapstain treated	Control	Anti-sapstain treated	Control	Anti-sapstain treated
Sphaeropsis sapinea	0.9	0.4	0.4	1.7	1.7	0	3.5	0.4	0	0	6.5	9.1
Total *Ophiostoma* species	32.2	27.9	0.4	1.7	70.4	17.9	7	5.7	85.7	25.4	13.9	13.9
Total sapstain isolates	76	68	2	8	166	43	24	14	197	61	47	53
Total number of wood pieces sampled	230	240	230	230	230	240	230	230	230	240	230	230

were also isolated from many of the different sites. Dowding[13] described these species as causing only surface discoloration, invading the wood more slowly than the *Ophiostoma* species and preferring dead sapwood with high food reserves. *Trichoderma* species were also isolated in both export trials.

There are very few reports of the *Ophiostoma* species in Japan and other Asian countries.[15] Both export trials showed that the sapstain fungi of New Zealand being exported to Japan are already present in that country. Aoshima[16] studied the wood-staining fungi of Japan and identified *S. sapinea*, *Leptographium* species, and members of the Ophiostomataceae family including the *Ophiostoma* species *O. floccosum*, *O. piceae*, *O. pluriannulatum*, *O. stenocerus*, *O. piliferum*, and *O. ips*. *Ophiostoma setosum*, *O. querci*, and *O. huntii* were not isolated in the study of Aoshima[16] but were isolated in both export trials. *Ophiostoma setosum* and *O. querci* belong to the *O. piceae* complex and until recently these fungi were difficult to distinguish from each other. *Ophiostoma huntii* has a *Leptographium* state and could have been isolated by Aoshima[16] and identified as a *Leptographium* species. No sapstain fungi that are considered serious pathogens (*Leptographium wagneri*, *O. ulmi*, *O. novo ulmi*, and *Ceratocystis* species) were detected on the logs and were not found to be transported from New Zealand.

The severity of stain and number of fungal isolations differed between the summer export trial and the winter export trial. The summer export trial was more heavily stained and more fungi were isolated. This is consistent with the results of Butcher,[5] who, while studying sapstain development on *Pinus radiata* posts above and below ground in New Zealand, found stain in almost all posts from December to May. Butcher[5] also compared the mean percentage staining with the monthly mean temperature and total rainfall and showed that the incidence of sapstain was closely associated with these factors. For both export trials, the temperature and rainfall or relative humidity were measured for the entire trial period. The increase in temperature and humidity while on the ship did not make a significant difference to the amount of sapstain and fungal colonisation for export trial 2 as indicated by the data. There was very little stain prior to shipment and the logs were still relatively sapstain free on arrival at the export destination.

Sapstain fungi were isolated from logs treated with antisapstain chemicals. The antisapstain chemical, which is applied to the log surface, kills the surface spores and hyphae but does not harm the sapstain fungi that have already penetrated into the wood. Additionally, sapstain fungi could have been isolated from the antisapstain chemically treated logs due to the presence of chemical-tolerant sapstain fungi. Xiao and Kreber[17] studied the effect of a chemical formulation of 3-iodopropynylbutylcarbamate/DDAC on spore germination and hyphal growth of *O. piceae*. Spore germination occurred within 24 h on untreated wood and the majority of spores did not germinate on treated wood. However, in some spores, the process of germination was delayed, but once it occurred, hyphae rapidly colonised the treated wood.[17]

Antisapstain chemicals used in New Zealand give only 8–10 weeks of protection.[3] The length of time from harvesting to arrival at the export destination for both trials was typical of export expectations and longer than the expected protection of the chemical. More effective methods of control are required if the delivery of sapstain-free logs requested by export customers is to be achieved.

This study showed the development and succession of sapstain fungi on logs from the time of harvesting to delivery at export destinations. No serious fungal pathogens were isolated in New Zealand or Japan. The effect of season on the development of sapstain was noted. Logs harvested in summer months were more prone to sapstain infection prior to antisapstain chemical treatment than in winter. The development of sapstain in logs felled in winter was not substantially increased by the hot, humid conditions encountered while crossing the equator. Consideration of the impact of season on the presence of sapstain fungi will also contribute to achieving delivery of sapstain free logs requested by export customers.

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