Towards a test to verify that wood has been heat-treated to the ISPM15 standard

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Abstract International trade of wood, including dunnage, is a well-known pathway for spreading diseases and wood-boring insects between countries. To mitigate this risk, the International Standard for Phytosanitary Measures No. 15 (ISPM15) states that wood packaging materials must be heated to a minimum of 56°C for 30 min. However, it is difficult to confirm that wood has been properly heat-treated. After preliminary investigations of 12 enzymes and 3 sugars from pine xylem, experiments focused on malate dehydrogenase (MDH) and fructose. Samples from the surfaces of heat-treated *Pinus radiata* wood exhibited decreased MDH activity and increased fructose concentration. However, samples from 5 mm deeper in the profile of heated wood showed similar MDH activity to unheated wood, but contained relatively lower fructose concentrations. There is potential to exploit heat-induced changes in MDH activity and fructose concentration to develop a quick, easily-used assay for verifying compliance of wood packaging materials with ISPM15.

Keywords heat treatment, ISPM15, *Pinus radiata*, xylem metabolites, malate dehydrogenase, fructose, wood packaging material.

INTRODUCTION

Wood packaging material (WPM), including pallets, crates and dunnage (used to brace cargo), is a primary pathway for accidentally moving bark beetles and wood-boring insects between countries (Brockerhoff et al. 2006; Zahid et al. 2008; Haack et al. 2014). Each year in the United States, wood-boring insects cost an estimated \$1.7 billion in local government expenditure and approximately \$830 million in lost residential property values (Aukema et al. 2011). They can also have irreversible impacts on native species and ecosystems (Poland & McCullough 2006).

To reduce biosecurity risks from WPM, the International Plant Protection Convention (IPPC 2002) developed a set of globally accepted measures called International Standards for

Phytosanitary Measures No. 15 (ISPM15). The standard stipulates that WPM must consist only of debarked wood to reduce risks of post-treatment reinfestation (Haack & Petrice 2009), although small pieces of bark may remain if they are less than 3 cm wide. WPM wider than 6 mm must be heat-treated or methyl bromide fumigated (IPPC 2013). Heating must reach a minimum temperature of 56°C throughout the wood profile, including the core, for a minimum of 30 continuous minutes (IPPC 2013). By October 2013, more than 78 countries had implemented ISPM15 (Haack et al 2014).

Upon treatment to the ISPM15 standard, WPM is stamped with a readily identifiable mark. National Plant Protection Organisations (NPPOs)

are responsible both for authorising use of the mark by treatment providers, and for regularly inspecting and auditing the providers' procedures (IPPC 2013). Sub-standard treatment, treatment failure and fraudulent use of the ISPM15 mark are all possible, thus a diagnostic test for verifying that WPM has been heat-treated in accordance with ISPM15 would be helpful for NPPOs. To develop such a test, it is necessary to identify one or more wood metabolites that consistently vary between unheated and heated wood.

Various enzymes and sugars have been detected in fresh wood samples (Sagisaka 1972; Haissig & Schipper 1978; Moeller et al. 2006), but there have been few studies of either dry or ISPM15-treated wood. In general, heating to 56°C is usually insufficient to completely inactivate enzymes, though it may reduce their activity (Anderson 2002; Gonzalez et al. 2011; Iline et al. 2013). Heating, however, should cause sap to move within xylem, thus metabolite concentrations in different parts of the wood profile may change with heat-treatment. Also, enzymes near the wood surface might receive elevated temperatures for longer than enzymes in the core, thus suffering greater thermal inactivation.

This paper investigates enzymes and sugars in xylem of *Pinus radiata* for their potential utility in verifying that WPM has been ISPM15 heat-treated. Methods similar to those recently applied to develop other tests for verifying compliance have been used (Iline et al. 2013; Novoselov et al. 2013; Phillips et al. 2013; Phillips et al. 2014).

MATERIALS AND METHODS

Two experiments were conducted. The first investigated the activity of 12 enzymes and the concentrations of three sugars in unheated *P. radiata* samples 0–2 weeks and 1–2 months after the wood was harvested. It also compared two sample maceration methods. Based on these results, the second experiment used one sample maceration method to compare malate dehydrogenase (MDH) activity and fructose concentration between unheated and heated samples. Experiment Two measured the metabolites both 1 month and 2 months after the wood was harvested. In the 2-month-old wood, samples were obtained from two locations in the

wood profile and compared. The enzymes and sugars chosen from the literature for investigation in Experiment One are listed in Table 1 with key references.

Pinus radiata collection, preparation and storage

Two branches (diameter 3–4 cm) were hand-sawn from each of five live *P. radiata* trees (5–7 m tall) about 1 m from the ground. The branches were cut into two 10 cm long segments and the bark was stripped from the distal end of each segment using a knife. Xylem was exposed by scraping the knife longitudinally along the exposed surface to remove the sticky cambium layer. Removing the cambium simulated milled timber, which usually also lacks cambium. The segments were labelled and stored in the laboratory, open to the air and at ambient temperature, from 1 week to 3 months prior to experiments.

Sample preparation

Wood shavings (30–50 mg/sample) were collected from the distal ends of 20 branch segments that had been stored for either 0–2 weeks or 1–2 months by scraping the sawn surface with a sharp knife to obtain fine shavings (< 1 mm). Liquid nitrogen (10–12 ml) was poured into a porcelain mortar, shavings were immediately added, liquid nitrogen was allowed to evaporate, then 0.1 M TAE buffer (pH 7.8) or 0.1 M Na-acetate (pH 5.0) (Table 1) was added to the mortar using an 11:1 ratio of extraction buffer volume (µl) to sample weight (mg), typically 330–550 µl. This was enough to dampen all the shavings.

The two sample maceration procedures compared in Experiment One involved either manually macerating each wood sample or using a bead mill. To manually macerate samples, the buffer-sample mixture was macerated with a porcelain pestle. For the bead mill method, the buffer-sample mixture was put into a specialised steel tube with either one or two 7 mm steel balls and shaken at 50 Hz for 1 or 2 min using a TissueLyser LT (Qiagen).

After maceration, samples were transferred by spatula into a 1.7 ml Eppendorf tube and centrifuged for 30 s at 9000 rpm. The clear supernatant (ca 70 µl) was transferred by pipette

into a 0.2 ml tube and kept chilled to minimise enzyme inactivation (Haissig & Schipper 1978). Supernatants were assayed within 1 h of centrifugation. For all assays, 7 µl of supernatant was added to an equal volume of stain (described below) in a 0.2 ml microtube (Axygen, California, USA, thin wall clear, PCR-02-C).

Activity staining of enzymes

Recipes for activity staining of most enzymes were modified from Manchenko (2003). Exceptions were activity stains for coniferyl and cinnamyl dehydrogenases, which used coniferyl alcohol or cinnamyl alcohol (Halpin et al. 1992; Sauter & Kende 1992; Pandey et al. 2011). To most recipes, 0.1% Triton X-100 was added, and NBT was replaced with MTT and PMS with mPMS (Phillips et al. 2013, 2014). Results were evaluated after staining for 30 min.

Evaluation of sugar concentration

Glucose, fructose and sucrose were assayed using methods based on Hoch et al. (2002). The glucose assay measures glucose only, but the fructose assay measures the combined concentration of fructose and glucose, and the sucrose assay measures the combined concentration of sucrose and glucose (Hoch et al. 2002). Preliminary tests indicated that glucose was present only in low concentrations in these samples so, for brevity, the latter two assays are referred to as the 'fructose test' and the 'sucrose test'. Recipes were modified by adding 0.1% Triton X-100 and replacing NBT with MTT and PMS with mPMS (Phillips et al. 2013, 2014). Results were evaluated after staining for 5 min.

Experiment One

The 12 enzymes and three sugars listed in Table 1 were each studied in a minimum of five samples chosen from 10 unheated *P. radiata* samples taken from two segments of five branches. Usually only metabolites that showed activity in 0–2 week-old samples were also assayed in 1–2 month-old samples. Sample maceration methods were compared using 3–5 samples per metabolite.

Experiment Two

Samples studied were from two 10 cm long segments from each of five branches collected

from five different live *P. radiata*. After 1 month of storage, one segment from each branch was heated in an incubator at 56°C for 1.5 h to simulate ISPM15, and the other was left as an untreated control. As the core temperature of the samples was unable to be measured, 1.5 h of heating was used to ensure the required core temperature was achieved. Immediately after heat-treatment, shavings were obtained from control and treated samples and prepared using the manual maceration method. Supernatants were tested for MDH and fructose.

Following a second month of storage, the same branch segments were re-tested for MDH and fructose. To compare the effect of heat treatment on metabolites in xylem situated at the wood's sawn surface with those situated below its sawn surface, a 5 mm thick section was hand-sawed off the distal end of each segment, then surface and sub-surface shavings were tested separately. Hand sawing was conducted slowly to minimise frictional heating of the timber.

Measurement of colour reaction

Photos were taken after 5 min for the fructose test, and after 30 min for the MDH test, then processed by ImageJ as described by Phillips et al. (2013). Optical density (OD) was used to estimate the intensity of the staining reaction (Luo et al. 2010).

Statistical analysis

Results from treated and untreated samples obtained from the same branch were compared using Student's t-test for paired samples with the statistical package R version 3.0.2 (R Core Team 2012). Graphs were prepared using R and the R package 'ggplot2' (Wickham 2009).

RESULTS

Experiment One

The manual sample maceration method gave similar sugar concentration results to the bead mill method, but higher enzyme activity (data not shown). Thus, results are provided only from manually macerated samples.

Four of the twelve enzymes tested, malate dehydrogenase (MDH), peroxidase, coniferyl and cinnamyl alcohol dehydrogenases, showed

activity in 0–2 week old samples, but MDH was the only active enzyme remaining in 1–2 month old samples (Table 1). Coniferyl alcohol dehydrogenase was not tested in 1–2 month old samples due to unavailability of chemicals (Table 1). Glucose, fructose and sucrose were all detected in samples of both ages, although fructose gave the most consistent and strongest staining. Thus, MDH and fructose were further investigated in Experiment Two.

Experiment Two

In the 1-month-old samples, the mean fructose concentration in heated samples (0.32 ± 0.03 ; SE) was higher than in untreated samples (0.18 ± 0.02 ; $t=7.278$, $df=4$, $P=0.002$) (Figure 1). In contrast, mean MDH activity was lower in

heated samples (0.11 ± 0.01) than in unheated samples (0.17 ± 0.01 ; $t=-6.742$, $df=4$, $P=0.003$) (Figure 1).

In 2-month-old samples taken from the same part of the wood profile as the 1-month-old samples, results were very similar. Mean fructose concentration was higher (0.37 ± 0.09 cf. 0.18 ± 0.02 ; $t=2.599$, $df=4$, $P=0.060$) and mean MDH activity lower in heated samples (0.13 ± 0.01 cf. 0.16 ± 0.03 ; $t=-2.426$, $df=4$, $P=0.072$) (Figure 2, 'External') compared with untreated samples. However, 2-month-old samples obtained from 5 mm deeper in the wood profile gave different results. Here, mean fructose concentration was lower in heated than untreated samples (0.14 ± 0.02 cf. 0.22 ± 0.04 ; $t=-3.118$, $df=4$, $P=0.036$), and mean MDH activity was higher

Table 1 Presence of enzyme activity and sugars in xylem of *Pinus radiata* tested at 2 weeks and 1-2 months after cutting. Markers were identified by a literature review.

Enzyme/sugar	EC No	buffer pH	Activity		Literature references
			0-2 wk	1-2 mo	
alcohol dehydrogenase	1.1.1.1	7.8	-	-	Mansell et al. 1976; Denslow & Hook 1986
malate dehydrogenase	1.1.1.37	7.8	+	+	Shain & Mackay 1973; Haissig & Schipper 1978
malic enzyme	1.1.1.40	7.8	-	-	Ryu & Na 1983
6-phosphogluconate dehydrogenase	1.1.1.44	7.8	-	-	Sagisaka 1972; Haissig & Schipper 1978; Long et al. 1994
glucose-6-phosphate dehydrogenase	1.1.1.49	7.8	-	-	Sagisaka 1972; Shain & Mackay 1973; Haissig & Schipper 1972, 1978
peroxidase	1.11.1.7	5.0	+	-	Campbell & Ellis 1992; Luo et al. 2012; Haissig & Schipper 1972, 1978; Marjamaa et al. 2006; Ryu & Na 1983
coniferyl alcohol dehydrogenase	1.1.1.194	7.8	+	-	Hawkins & Boudet 2003; Li et al. 2012; Luo et al. 2012
cinnamyl alcohol dehydrogenase	1.1.1.195	7.8	+	-	Anterola & Lewis 2002; Bedon et al. 2009; Campbell & Ellis 1992; Guo et al. 2010; MacKay et al. 1999; Moeller et al. 2006; Ramos & Herrera 2013; Saralde et al. 2008
β -D-glucosidase	3.2.1.21	5.0	-	-	Campbell & Ellis 1992; Samuels et al. 2002
β -D-glucuronidase	3.2.1.31	5.0	-	-	Bedon et al. 2009
glucose-6-phosphate isomerase	5.3.1.9	7.8	-	-	Long et al. 1994
acid phosphatase	3.1.3.2	5.0	-	-	Galimova 1987
glucose ¹	n/a	7.8	+	+	Fischer & Hoell 1991, 1992; Hoch
fructose ¹	n/a	7.8	+	+	et al. 2002, 2003; Stasova &
sucrose ¹	n/a	7.8	+	+	Antonova 1989; Wang et al. 2009

¹Same references for all three sugars.

Figure 1 Box plots showing optical densities of tests for fructose concentration and malate dehydrogenase activity in heated and unheated samples taken from the sawn surfaces of wood. The wood had been stored for 1 month. Lower and upper horizontal lines in each box show first and third quartiles, respectively, and the dark central line shows median. Whiskers extend to the highest and lowest values that are within 1.5 times the distance between the first and third quartiles. Diamonds show means and 'x' shows outliers.

Figure 2 Box plots showing optical densities of tests for fructose concentration and malate dehydrogenase activity in heated and unheated samples taken from the sawn surfaces of wood ('External') and from 5 mm below sawn surfaces ('Internal'). The wood had been stored for 2 months. Lower and upper horizontal lines in each box show first and third quartiles, respectively, and the dark central line shows median. Whiskers extend to the highest and lowest values that are within 1.5 times the distance between the first and third quartiles. Diamonds show means and 'x' shows outliers.

in heated (0.18 ± 0.01) compared with untreated samples (0.17 ± 0.01 ; $t=2.450$, $df=4$, $P=0.070$) (Figure 2, 'Internal').

Some ratios between optical densities obtained from different tests and samples clearly separated heated and unheated wood. Using samples taken from sawn surfaces of 1-month-old wood, for example, the mean ratio of MDH activity to fructose concentration was higher in untreated samples (1.02 ± 0.17) than in heated samples (0.38 ± 0.18 ; $t=-5.834$, $df=4$, $P=0.004$) (Figure 3a). Alternatively, fructose concentration measured from sawn surfaces of 2-month-old wood divided by fructose concentration measured from 5 mm below sawn surfaces of 2-month-old wood was always lower in untreated samples (mean 0.83 ± 0.08) than in heated samples (2.65 ± 0.56 ; $t=3.4235$, $df=4$, $P=0.027$) (Figure 3b).

DISCUSSION

Changes in enzyme activity and sugar concentration in heat-treated wood compared to controls were observed, indicating there is potential to produce an assay for verifying compliance of WPM with ISPM15. A relatively simple sample preparation procedure enabled

MDH activity and fructose concentration to be compared between samples, even in wood stored for 2 months. The equipment required is common to most laboratories and the results were reproducible and quantifiable.

Samples macerated in a bead mill gave weaker enzyme activity staining than those macerated manually, possibly because collisions between the steel balls in the bead mill heated the samples and contributed to enzyme inactivation. The sample preparation method using manual maceration of wood ensured samples remained cold, which enabled testing both of enzymes and sugars. This is unlike previous wood sample preparation methods that preclude most enzyme tests because they involve heating (Hoch et al. 2002; Wang et al. 2009).

Activity was detected in only one of twelve enzymes investigated in these *P. radiata* samples, which had been stored for up to 2 months. Other studies have found activity of additional enzymes in wood (cited in Table 1), but these were always obtained from fresh wood samples. It appears that MDH activity is relatively persistent in stored wood; thus has potential for testing the compliance of WPM with ISPM15.

Figure 3 Box plots showing (a) ratio of MDH activity to fructose concentration measured in heated and unheated samples obtained from sawn surfaces of 1-month-old wood, and (b) ratio of fructose concentration measured from sawn surfaces of 2-month-old wood to fructose concentration measured from 5 mm below sawn surfaces of 2-month-old wood. Lower and upper horizontal lines in each box show first and third quartiles, respectively, and the dark central line shows median. Whiskers extend to the highest and lowest values that are within 1.5 times the distance between the first and third quartiles. Diamonds show means and 'x' shows outliers.

Malate dehydrogenase activity was suppressed by the simulated ISPM15 treatment (56°C for 1.5 h) more in samples obtained from the wood surface than in samples obtained from 5 mm beneath it. Possibly it took some time for the whole wood profile in the samples to reach 56°C, with the surface being quickest to heat, thus explaining why MDH activity was weakest at the surface. Partial inactivation of MDH at 56°C has also been observed in yeast (Kohda et al. 2006).

Compared to controls, samples from the external surfaces of heated wood contained higher concentrations of fructose. Lambertz & Welling (2010 a,b) also observed increased sugar concentrations on the surface of heated pine wood. The higher fructose concentrations found in xylem on the sawn surface of heated wood than in xylem situated 5 mm below it might be explained by sap movement. Heating probably causes sap to move through xylem towards the ends of branches, thus producing higher concentrations of sap ingredients at sawn surfaces. WPM treated in steam chambers is highly susceptible to infection by moulds and other fungi if not adequately dried before storage due to heat-induced migration of simple sugars to the surface (Zahid et al. 2008).

Xylem comprises membranes with nanoscale pores (Boutillier et al. 2014). The large difference in molecular weight between fructose (180) and MDH (ca 70000) may help explain why fructose concentration, but not MDH activity, increases at the wood surface after heating. Low molecular weight simple sugars such as fructose would probably migrate more quickly than enzymes through xylem pores.

These results indicate that further research to develop a compliance test for ISPM15 would be worthwhile. Future work would need to investigate a wider range of wood species because ISPM15 is applicable to wood of any species, including tropical to boreal species, softwoods (conifers) and hardwoods (angiosperms) (Haack et al. 2014). It would also need to consider the influences of seasonal fluctuations in wood metabolites. For example, sugars in other *Pinus* spp. can vary with air temperature, water supply (Stasova & Antonova 1989) and season (Campbell

et al. 1999; Hoch et al. 2002). Similarly, MDH activity varies seasonally in *P. radiata* (Shain & Mackay 1973). The simulated ISPM15 treatment was adequate for this initial research, but future studies should use wood commercially treated to ISPM15 standard using longer intervals between treatment and testing. This should include woods heated via the different processes allowable under ISPM15, which include kiln-drying, heat-enabled chemical pressure impregnation and microwave radiation (IPPC 2013).

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