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Etiology of Red Stain in Boxelder

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Abstract

Fusarium reticulatum (*F. negundi*) has been suggested as the cause of red stain in boxelder (*Acer negundo*), but the role of the fungus in producing the pigment has never been confirmed. This study elucidated the cause of red stain using a series of wounding and inoculation experiments. Fungal colonization and stain development were examined in 20 wounded boxelder trees at 8, 12, 52, and 64 weeks after wounding. *Fusarium solani* was isolated from 67 to 83% of the stained, wounded tissue. Boxelder trees were inoculated in the field with *F. acuminatum*, *Hypsizygus tessulatus*, or left not inoculated to serve as the control. Eight weeks after inoculation, *F. solani* was isolated from 67, 17, and 33% of the wounds treated with *F. acuminatum, H. tessulatus*, and the control, respectively. All wounds had typical red stain 12 weeks after inoculation including those inoculated with *H. tessulatus* and the control. *Fusarium reticulatum* was not isolated from any wounded or inoculated boxelder tree. The stain's ubiquitous presence in all wounded tissue and the inability of *F. solani* isolates obtained from boxelder to stain boxelder red in wood block studies indicates that red stain is most likely produced by the tree as a nonspecific host response to wounding.

Introduction

A ubiquitous red stain can be found in the sapwood of living boxelder (*Acer negundo* L.) throughout the range of the species. Because the stain is so frequently encountered, it is often considered to be a reliable characteristic for identifying boxelder wood (3,7). It has been reported to occur in the wood within the main stem, in roots greater than 5 cm in diameter and in branches greater than 2.5 cm in diameter (Fig. 1) (6). Pigmentation is localized around branch scars, frost cracks and wounds caused by insects or animals, such as sapsuckers (*Sphyrapicus varius*) (3,7). It is thought that these localized areas of discolored wood coalesce over time creating a large, central stained column within the tree (Fig. 2). Colors range from light pink to dark red, but the stain fades over time when exposed to light. Red stain in boxelder is of special interest to woodworkers who use the attractive stained wood for decorative purposes (Fig. 3). The stain does not appear to cause significant loss of structural integrity in the sapwood (6).

Fig. 1. Cross-section of a central column of red stain in boxelder.

Fig 2. Red stained pockets from wounds and branch scars coalesce to form a central red column.

Fig. 3. A vase made out of red stained boxelder.

Fusarium reticulatum Mont., formerly known as *F. negundi* Sherb. and *F. reticulatum* Mont. var. *negundinis* (Sherb.) Wollenw., has been reported as the causal agent of red stain despite the fact that researchers have been unable to reproduce the stain by inoculating boxelder wood blocks with *Fusarium* spp. (3,6,7,10). Moreover, Hubert (6), who first isolated and reported *F. reticulatum* as the causal agent, later questioned his own findings (7). The ubiquitous presence and the unique discoloration pattern of red stain in boxelder suggest that some other agent may be responsible for the stain. For example, a discoloration pattern similar to that in boxelder occurs in sugar maple (*A. saccharum* Marsh.) in the absence of fungi as a result of bird pecks, nails driven into trees, and injections with distilled water and copper sulfate (5). The pattern of staining in boxelder is also similar to the discoloration observed in many other species of deciduous trees (2,15). Therefore, this study was undertaken in an attempt to elucidate the true cause of red stain in boxelder.

Red Stain and Microorganisms Associated with Boxelder Wounds

In June 2000, 20 mature boxelder trees in a mixed hardwood stand in southeastern Minnesota were selected for wounding. Six wounds per tree were made by drilling holes 1.6 cm in diameter and 4.4 cm deep. The wounds were made at 20-cm intervals in a spiral pattern up the bole of the tree beginning approximately 30 cm above the ground. No red stain was evident when trees were wounded. Four trees were felled and examined after 4, 8, 12, 52, and 64 weeks. The logs were split longitudinally and small segments of red stained

wood were aseptically removed from above and below the wounds and placed on Difco malt extract agar (MEA 1.5%) and Nash-Snyder select medium (PCNB) for *Fusarium* spp. (4,10). Using the methods outlined by Nelson et al. (10), cultures were identified after single-spore isolates were grown on carnation leaf agar (CLA) and potato dextrose agar (PDA) (4).

Four weeks after trees were wounded in the field, the incipient stages of stain ranging from dark tan to light pink were present above and below all wounds. *Fusarium solani* was isolated from 75% of the wounded tissue (Table 1), no microorganisms were isolated from 21% of the wounded tissue, and 4% of the wounded tissue was colonized by other microorganisms. By 8 weeks, orange to carmine stains extended approximately 1 cm below and above all wounds. After 12 weeks, carmine stain was associated with all wounds and extended on average 3.0 cm below and 2.8 cm above the wounds. The amount and color of staining did not change from the fall (12 weeks) to the spring (52 weeks), but by the end of the second growing season (64 weeks), the stain extended on average 9.2 cm below and 9.5 cm above all wounds. *Fusarium solani* was isolated from 83, 83, 82, and 67% of the wounded tissue at 8, 12, 52, and 64 weeks, respectively. *Fusarium reticulatum* was not isolated.

Table 1. Frequency of microorganisms recovered from stained boxelder wood associated with artificial wounds 4, 8, 12, 52 and 64 weeks after wounding.

	Percent[a]			
Week harvested	***F. solani***	**Unidentified basidiomycete**	**Other species**	**No fungi isolated**
4 (n=24)	75	12	17	21
8 (n=24)	83	8	17	4
12 (n=24)	83	8	0	12
52 (n=22)	82	4	18	0
64 (n=24)	67	0	33	0

[a] Microorganisms recovered are reported as a percentage of the total number of wounds sampled (n). More than one species was often collected from a single sample.

Red Stain and Microorganisms Associated with Inoculations

Sample logs from 52 red-stained boxelder trees were split longitudinally and small segments of stained wood were aseptically removed and placed on Difco MEA (1.5%) and PCNB media. Of the 149 cultures obtained from stained wood, only one isolate resembled *F. reticulatum*. This isolate was sent to Dr. W.F.O. Marasas (PROMEC Unit, Medical Research Council, Tygerberg, South Africa) who identified the isolate as *F. acuminatum* Ell. & Ev. (*personal communication*). The *F. acuminatum* isolate was also examined using amplified fragment length polymorphism (AFLP) fingerprinting and was found to be > 80% similar in fingerprint profile to *F. reticulatum* isolates R-0118 and R-5186 obtained from the Fusarium Research Center (Pennsylvania State University). *Fusarium reticulatum* isolate NRRL 20682 obtained from the USDA Agriculture Research Service (ARS) culture collection was > 30% similar to the *F. acuminatum* isolate by unweighted pair group method with arithmatic mean (UPGMA) estimated band sharing (K. Zeller, Kansas State University, Manhattan, KA. *Personal communication*). Except for NRRL 20682, these species are apparently related based on AFLP fingerprinting (11).

The *F. acuminatum* isolate obtained from red stained boxelder and *Hypsizygus tessulatus* Bull ex Fr. were grown on autoclaved oats for 5 weeks at 24°C to be used as inoculum for field studies. *Hypsizygus tessulatus* (Fig. 4), a common decay fungus in boxelder, was included in the study to determine if the fungus affected stain development, and conversely, if red stain altered the growth of the decay organism. Autoclaved oats were used as a control. In June

2000, holes 1.6 cm in diameter and 4.4 cm deep were drilled into 24 mature boxelder trees from the same hardwood stand used in the wounding study. No red stain was evident when holes were made. Two holes on each tree were inoculated with the *F. acuminatum* isolate obtained from red stained boxelder, two holes were inoculated with *H. tessulatus* and two were filled with autoclaved oats for a total of six holes spirally arranged in the bottom 2 m of the tree. Treatments were systematically distributed at different heights in the trees to avoid any effect wound position might have had on colonization ability.

Fig. 4. Fruiting bodies of *Hypsizygus tessulatus*.

Fig. 5. The apparatus adapted from Sharon and Shigo (14) used to cover inoculated holes. Rubber tubing filled with sterile cotton was substituted for a test tube. The apparatus enables oxygen to circulate but prevents contamination.

Two wound coverings were used. One wound of each treatment was covered with duct tape. The other wounds were plugged with a cork stopper with a glass rod running through it. The rod was attached to rubber tubing that had been packed with cotton at both ends (Fig. 5). Hot glue was applied around the junction of the glass tube and the cork to prevent possible contamination of a wound by airborne fungal or bacterial propagules. Each apparatus was autoclaved for 20 min at 121°C before being inserted into a hole and sealed with duct seal. The apparatus was modified from Sharon and Shigo's (14) wound covering in which a glass test tube filled with autoclaved cotton and paraformaldehyde tablets was used instead of rubber tubing. With either apparatus, oxygen was able to circulate in the wound while keeping the treatments free from contamination.

Six trees were felled 8, 12, 16, and 52 weeks after inoculation and examined for microbial colonization and red stain development. Small sections of red stained wood were placed on MEA (1.5%) and PCNB media (4). Identification of Fusarium isolates followed the methods described above.

Throughout the field inoculation study there were no noticeable differences in staining between wounds covered with either the duct tape or the cork apparatus. Eight weeks after inoculation, the incipient stages of stain ranging from dark tan to light pink were seen above and below all wounds. *Fusarium solani* was isolated from 67, 17, and 33% of the wounds covered with a cork apparatus and inoculated with *F. acuminatum*, *H. tessulatus*, and the control, respectively (Table 2). Even though *H. tessulatus* and control wounds covered by the cork apparatus were stained 8 weeks after inoculation, no microorganisms were isolated from 67% of the stained wood from these treatments. All tissue surrounding inoculated wounds was stained red 12 weeks after inoculation

including those inoculated with *H. tessulatus* and the control (Fig. 6). At 52 weeks, *F. solani* had colonized 80, 83, and 83% of the red stained wood in cork-covered wounds inoculated with *F. acuminatum*, *H. tessulatus*, and the control, respectively (Table 3). *Fusarium reticulatum* and *H. tessulatus* were never reisolated. Microscope examinations of the stained wood showed pigmented cells that were free of hyphae.

Table 2. Frequency of microorganisms recovered from stained boxelder wood 8 weeks after inoculation with *F. acuminatum*, *H. tessulatus*, or the control.

	Percent[a]		
Treatment	***F. solani***	**Other species**	**No fungi isolated**
F. acuminatum			
Tape (n=6)[b]	83	33	0
Cork (n=6)	67	17	17
H. tessulatus			
Tape (n=5)	60	0	40
Cork (n=6)	17	17	67
Control			
Tape (n=6)	50	0	50
Cork (n=6)	33	0	67

[a] Fungi recovered are reported as a percentage of the total number of wounds sampled (n). More than one species was often collected from a single sample.

[b] Inoculations were either covered with duct tape or sealed with a cork apparatus.

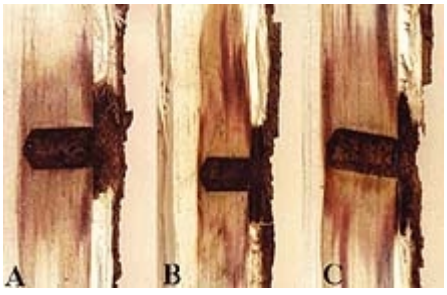

Fig. 6 Red stain in boxelder 12 wks after inoculation with (A) *F. acuminatum*, (B) *H. tessulatus*, and (C) control.

Table 3. Frequency of microorganisms recovered from stained boxelder wood 52 weeks after inoculation with *F. acuminatum, H. tessulatus*, or the control.

	Percent[a]		
Treatment	***F. solani***	**Other species**	**No fungi isolated**
F. acuminatum			
Tape (n=6)[b]	83	17	0
Cork (n=5)	80	0	20
H. tessulatus			
Tape (n=6)	50	0	50
Cork (n=6)	83	0	17
Control			
Tape (n=6)	83	0	17
Cork (n=6)	83	17	0

[a] Fungi recovered are reported as a percentage of the total number of wounds sampled (n).

[b] Inoculations were either covered with duct tape or sealed with a cork apparatus.

Wood Block Inoculation Studies

The *F. acuminatum* isolate obtained from red stained boxelder and *F. reticulatum* isolates (R-0118, R5186, NRRL 20682) were examined in the laboratory for staining ability. Wood blocks 1.6 cm x 1.6 cm x 1.9 cm were cut from a boxelder tree harvested for this purpose. The blocks were soaked in water for 18 h, placed in jars with 10 ml vermiculite and 6 ml of water and autoclaved at 121°C twice for 60 min each. The *Fusarium* isolates were grown on potato dextrose broth (10) for 1 week after which fungal mats 1.5 cm in diameter were placed on top of the autoclaved wood blocks and incubated at 27°C and 85% relative humidity. Four replicates were prepared for each isolate and non-inoculated wood blocks served as the control. Six weeks after wood blocks were inoculated with *F. acuminatum* and *F. reticulatum* isolates, no stain resembling that found in nature was observed. *Fusarium reticulatum* isolates R5186 and NRRL 20682 lightly stained the wood blocks a pinkish gray. *Fusarium acuminatum* and *F. reticulatum* R-0118 produced localized purple areas that did not extend into the wood block.

The ability of *F. solani* isolates to stain boxelder wood was examined by inoculating boxelder wood blocks in the laboratory. The wood blocks were prepared following the methods described above. Four isolates of *F. solani* obtained from red stained boxelder wood were grown on potato dextrose broth for 3 weeks, after which the fungal mats were placed on top of the autoclaved wood blocks. Wood blocks were incubated at 27°C and 85% relative humidity for 3 months. Ten replicates were prepared for each isolate and for the non-inoculated control. Two months after wood blocks were inoculated, *Fusarium solani* was unable to produce the red stain found naturally in boxelder. Instead wood blocks were stained a light purple.

The ability of *F. solani* and *H. tessulatus* to colonize boxelder sapwood, red-stained wood, and decayed wood was determined by inoculating wood chips in the laboratory. Sapwood chips, red-stained wood chips, and decayed wood chips approximately 1 cm x 3 cm were collected from a boxelder tree harvested for this purpose. Ten to 15 wood chips were placed in petri plates which were then autoclaved twice at 121°C for 60 minutes each and inoculated with 4 plugs from either *F. solani* or *H. tessulatus* isolates grown on PDA and MEA, respectively. Three replicates were prepared for each isolate and for the non-inoculated

control. After two months, *F. solani* was only able to colonize, but not stain, sapwood chips whereas *H. tessulatus* was only able to colonize decayed wood chips. Red stained wood chips were not colonized by either fungus.

Pigment Analysis of Stains

The pigments produced by *F. solani* inoculated onto wood blocks were extracted with methanol in a Soxhlet apparatus and compared to the pigments extracted by the same method from naturally occurring red stain (12). The extracted pigments were concentrated using a vacuum rotary evaporator and then suspended in chloroform:methanol (4:1 v/v). The pigments were spotted onto thin layer silica gel plates and developed with chloroform:methanol (97:3 v/v). The extract from *F. solani* stained wood blocks was composed of different pigments than the extract from naturally occurring red-stained boxelder. The R_f-values (polarity) for the various pigments produced by *F. solani* were measured and found to be different than the pigments produced in naturally occurring stain indicating that they consisted of different compounds (Table 4).

Table 4: R_f-values for pigments extracted from *F. solani* inoculated wood blocks and naturally occurring red stain using thin-layered chromatography developed with chloroform:methanol (97:3 v/v).

Pigments present in extracted samples	*F. solani* extract	Naturally occurring boxelder red stain extract
Pink	0.670	0.804
Purple	0.959	-----[a]
Blue	0.330	0.706
Yellow	-----	0.978

[a] Not present

Discussion

Although previous investigators have suggested *F. reticulatum* to be the cause of red stain (3,6,7,18), the fungus was never isolated from stained boxelder wood in this study. The only isolate obtained in this study that resembled *F. reticulatum* was identified as *F. acuminatum*. *Fusarium acuminatum* is morphologically similar to *F. reticulatum* in that microconidia are not produced. In addition, both species are slow-growing and produce white mycelia and a burgundy pigment when grown on PDA. A main difference between the two species is the morphology of the macroconidia. Although both species produce slender macroconidia, those of *F. acuminatum* tend to have longer, more tapering apical cells and a distinct dorsi-ventral curvature. Furthermore, macroconidia of *F. acuminatum* are usually 5 septate whereas *F. reticulatum* are typically 3 septate (10).

It is impossible to verify the identification of the original isolates of *F. reticulatum* used by Sherbakoff (6) because cultures are not available in any collection. In contrast to the description of *F. reticulatum* by Nelson et al. (10), Sherbakoff (6) described the isolates as fast-growing and bearing five-septate macroconidia with curved, attenuate apex cells. Most noteworthy, he reported that microconidia were formed whereas *F. reticulatum* does not produce microconidia (10). However, *Fusarium* morphology is dependent on the type of medium used and Sherbakoff's isolates were grown on oat and prune agar instead of PDA and CLA. Therefore, it is not known if the isolates were correctly identified as *F. reticulatum*. Because Sherbakoff's original cultures were unobtainable, the staining ability of *F. reticulatum* isolates (R-0118, R5186, NRRL 20682) from the Fusarium Research Center and USDA ARS culture collections were examined instead. None of the isolates from the culture collections were able to produce a stain similar to that found in nature.

Hypsizygus tessulatus was also not involved in stain production. In laboratory studies, *H. tessulatus* was only able to colonize decayed wood chips

indicating that sapwood and red stained wood are not suitable substrates for *H. tessulatus*.

After 8 weeks, 67% of the *H. tessulatus* and control treatments were stained the same amount and intensity as wounds inoculated with *F. acuminatum*, however, no microorganisms were isolated. Toole (18) also obtained stained controls when inoculating boxelder with *F. reticulatum*. This evidence, along with the conflicting R_f-values and the inability of *F. solani* to stain boxelder wood red, indicates that *F. solani* is not the causal agent but is a primary colonizer of wounded tissue. *Fusarium* spp. are normal wound colonizers in many tree species. For example, *Fusarium* spp. were one of the most frequently isolated fungi from basswood (*Tilia americana* L.), sugar maple (*A. saccharum*), yellow birch (*Betula alleghaniensis* Britton), and paper birch (*Betula papyrifera* Marsh.) when wounded with an increment borer (9). Shigo (17) reported that 26% of isolates obtained from wounded red maple (*A. rubrum* L.) were *Fusarium* spp. 6 months after trees were wounded. It is possible that higher percentages of *Fusarium* would have been obtained if a selective medium, such as Nash-Snyder selective medium (4), had been used. The high percentages of *F. solani* colonization in boxelder may also be due to the time of year when trees were wounded. Shain and Miller (13) isolated *F. solani* significantly more often from trees wounded during the growing season than during the dormant season. It is also possible that boxelder provides a unique habitat for *F. solani*.

Efforts have been made to characterize the chemical composition of the red pigment in boxelder; however, this has not been possible due to the instability of the compound (12). If the compound is a phenol, as we suspect, the oxidation of wounded cells may explain the localized areas of red stained wood frequently found around wounds and branch scars in boxelder (8,16). It is also possible that microorganisms, such as *F. solani*, stimulate the tree's natural host response to elicit the chemical reactions in the wounded tissue (15,16). Similar host responses in the presence of *F. solani* have been reported in other tree species. In yellow poplar (*Liriodendron tulipifera* L.), a dark brown pigment, presumably caused by the oxidization of a phenol, was produced by the tree in wounded cells and in response to *F. solani* (1). Although the brown pigment did not prohibit *F. solani*, it did restrict its growth. It is possible that the red stain in boxelder is produced by the tree to restrict growth of invading fungi such as *F. solani*. For example, both *F. solani* and *H. tessulatus* were unable to colonize red stained boxelder wood chips in laboratory studies.

The results from this study provide evidence that red stain in boxelder is not caused by *F. reticulatum* as previously recorded in the literature. Instead, the stain appears to be produced by the tree as a non-specific host response. Future research is needed to characterize the chemical properties of the pigment in order to ascertain its structure and function. In addition, researchers should examine the biological activity of the pigment to better understand how the compound influences microbial colonization of boxelder. Future research should also determine how microorganisms, such as *F. solani*, might stimulate and enhance pigment production.

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