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Recent researches on Sapstaining Fungi Colonizing Pines

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During last decade there has been noticeable progress in the research of the biology of sapstaining fungi that cause considerable economic losses to forest product industry. The researches generated broad ranges of knowledge on sapstaining fungi regarding their occurrence on conifer wood, taxonomy, nutrient physiology, pigmentation biochemistry and molecular biology, and biological control. Major problematic groups in the sapstain production are *Ophiostoma*, *Ceratocystis*, and *Leptographium* genera. With *Ophiostoma* as a model, it is found that the type of carbon source is important in the growth and pigment production of sapstaining fungi. The operation of dihydroxy naphthalene (DHN) melanin pathway for black to bluish pigment production has been confirmed in those cosmetic fungi both at biochemical and molecular levels. The development of albino technology using nutrition competition has been shown to be promising as an environmentally friendly biological control method for sapstain control.

Keywords : albino technology, biocontrol, *Ceratocystis*, DHN melanin pathway, *Leptographium*, *Ophiostom*, sapstain

With the globalization of forest industry and export of wood products, there is a high risk to introduce exotic pests to native forests of trading partners. Introduction of exotic pests such as fugal pathogens can cause catastrophically and irreversibly damage to the new forest ecosystem. For example, in twentieth century, Europe and then North America experienced two major epidemics of Dutch Elm Disease caused by *Ophiostoma ulmi* and *O. novo-ulmi*. Following introduction of these Asian bark-beetle fungal symbionts, 30 million Elm trees were killed in Britain and most of Elm trees were killed in North America. It is important to know that most of the bark-beetle associated fungi belong to the same *Ophiostomatoid* group.

Some of the *Ophiostomatoid* fungal species are saprophyte or weak pathogens but some are highly pathogenic and considered as the quarantine pests. A few years ago, the United States referred to *Ophiostoma* as the subject of phyto-sanitary regulation. Similar to the United States, some European countries and Australia are conducting a risk assessment on imported wood products that are suspected to harbour *Ophiostoma* and decay fungal species. *Ophiostoma* genus is one of well known fungal groups that cause stain in the wood of tree and fresh logs. Species belonging to this sapstain genus do not cause structural damages but cause the blue-black discoloration of sapwood (Fig. 1). The wood discoloration caused by *Ophiostoma* fungi is cosmetic damages and a worldwide problem in the forest products industry since it reduces the value of wood (Seifert, 1993). In spite of its importance, however, there has been a very poor data set about the biology of this group of fungi and their interaction with conifer hosts. To protect pine trees and wood from the problematic organisms, generation of information on the organism is prerequisite.

Fig. 1. Discolored pine sawood area colonized by sapstain fungi.

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Thus last decade researches have been performed on the occurrence, taxonomy, nutrient physiology, pigmentation biochemistry and molecular biology, and biological control of sapstain fungi. This review has focused on recent advance in Canadian sapstaining research, because evident progress has been shown in Canada.

Mycoflora of sapstained conifer wood

To determine which fungi cause stain problem, a detailed survey was conducted at seven selected sawmills across Canada in 1997. From five important conifer species including *Abies balsamea, Picea mariana, Picea glauca*, and *Pinus contorta*, over 2000 fungal isolates were isolated and identified based on morphological and physiological properties and mating compatibility (Fig. 2). Five genera and 13 species were found. *Ophiostoma* was the most commonly encountered genus (97%). A more diverse range of fungi was found in logs than lumber (Uzunovic et al., 1999). From the survey, *Ophiostoma setosum* was identified as a new species (Uzunovic et al., 2000). *O. piceae* was most dominant species and could be differentiated from sibling species, *O. quercus* by PCR (Kim et al., 1999). Consequently, common ITS rDNAs shared by *O. piceae* and *O. quercus* were found (Kim and Breuil, 2001).

To investigate whether or how the occurrence of stain varies depending on season, a seasonal survey of staining fungi was conducted in a saw mill in Princeton in British Columbia province in 1998, using fresh lodgepole pine logs and lumber. 510 isolates were obtained from samplings in spring, summer, and fall. They distributed in five different genera representing at last 10 species. Overall, the results showed that the major sapstain species (*Leptographium* spp., *O. floccosum*, and *O. piceae*) occur throughout different seasons. Freshness of wood is considered being attributed to the variance in their occurrence ensuing deviation in stain in different species (Uzunovic et al., 2000).

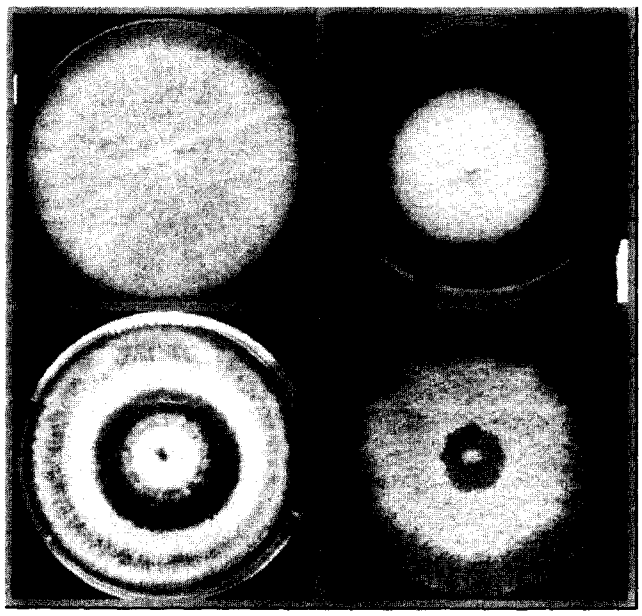

Fig. 2. Colony morphology of some sapstaining fungi.

Penetration tests of pine

The penetration of sapstain fungi appears to involve interruption of water conduction in sapwood, even though the mechanism is not yet clear. It has been shown that the disruption of water conduction occurs in wedge shaped zone ahead of the stained sapwood and in fungal colonized area. This fungus free zone is 0.1 to 5 mm wide and has been demonstrated by histological methods (Sangwon Lee, unpublished data).

Ophiostoma fungal vector

Bark beetles are well known in conifers as one of important fungal vectors (Solheim, 1986). The relationship between these vectors and sapstaining fungi has not much been studied except few sapstaining fungi. Major concern has been done to the species that are pathogenic and cause sapstain on pines. Beetle-fungal infestation including Mountain pine beetle, spruce beetle and engraver beetle in Northern British Columbia is a critical issue that imposes serious economic burdens on the province. Mountain pine beetle (MPB) carries staining fungi externally, as well as internally in their mycangia. *O. pulvinisporum, O. clavigerum* and *O. montium* are found pine trees associated with MPB (Zhou et al., 2003). Within a tree, the beetles remain in the region just under the bark, mining the phloem, while the staining fungi propagate in beetle galleries and the underlying sapwood. The vectored staining fungi benefit because the beetles carry them through the tree bark, making available a fresh, moist, nutrient-rich wood environment with no competing microflora. The beetles benefit because the fungi 1) make the attacked environment more favorable for the beetles by lowering the wood moisture content, 2) weaken tree defense mechanisms against the attacking beetles by releasing chemicals that kill parenchyma cells, and 3) make nutrients available (particularly nitrogen) that the beetles need to complete their life cycle. It has been shown that *O. clavigerum* and *O. montium* are necessary for successful insect brood development (Diana, 2003).

Phylogenetic and population genetic studies

Phylogenetic analysis was approached initially using rRNA

gene sequence (Hausner et al., 1993). With extensive preliminary test of universal primers (Kim et al., 1998), the PCR-amplified rDNA regions of the 18S and partial 26S subunit rRNA genes, the 5.8S rRNA gene, and internal transcribed spacers (ITS) 1 and 2 were sequenced from many sapstaining species. All the *Ophiostoma* species were clearly separated from the out-groups species, *Ceratocystis* species and *Neurospora crassa*. The phylogram generated by the sequence analysis of the 18S rRNA gene showed that *O. piliferum* was closely grouped with *O. penicillatum* and *O. bicolor*, and subgrouped with *O. ulmi*, *O. quercus*, and *O. floccosum*. In this phylogram, *O. cucullatum* and *O. europhiloides* were far apart from *O. piliferum*. Similarly, in the 26S rRNA gene-based phylogram, *O. piliferum* was grouped closely with *O. bicolor*, neighborly with *O. floccosum*, and distantly with *O. cucullatum* and *O. europhiloides*. The ITS-based phylogram further supported the distance relationship of *O. piliferum* to *O. cucullatum*. PCR-RFLP makers for differentiating these fungi were developed using rDNA region as target sequences (Kim et al., 1999, 2000). Then relationship of *O. minus* and *O. ips* related species could be resolved using β-tubulin genes (Kim et al., 2003, 2004)

To generate information on the genetic diversity of *O. piliferum* in Canada, genetic variability of a sample of 65 isolates of *Ophiostoma piliferum*, recovered in 1997 from softwood lumber at sawmills located in four Canadian provinces (Alberta, British Columbia, Ontario, Saskatchewan), was assessed by RAPD-PCR. Allelic frequencies at 163 putative RAPD loci were computed. Genetic diversity within geographic populations (H(S)) was estimated at 0.220 and accounted for only 75% of total diversity (H(T) = 0.310). Thus, approximately 25% of the overall diversity was due to frequency differences among populations (G(ST) = 0.240), suggesting the occurrence of high level of differentiation among the four populations studied. Further comparisons indicated that populations from British Columbia and Alberta and from Saskatchewan and Ontario were much closer genetically, resulting in a marked difference between the two western and the two central Canadian populations. These results suggest that the Canadian populations of *O. piliferum* are genetically more differentiated than comparable populations of the related species *O. piceae*, for which a G(ST) of 0.112 was previously estimated based on a similar methodology (Bernier et al., 1998). Overall, these results suggested that *O. piliferum* is highly heterogeneous with a continuously distributed population across Canada (Bernier et al., 2001).

Physiology of sapstaining fungi on wood

Fungal growth. Fourteen strains were inoculated in pine logs. The fastest growing species was *C. coerulescens* (> 30 cm longitudinal stain in 28 days), followed by *Leptographium* spp (14-18 cm), *O. minus* (4-11 cm), *O. piliferum* (2-5 cm), *O. piceae* (1-4 cm), *O. floccosum* (2.5-4 cm), *O. setosum* (2-3 cm), *O. species E* (1-3 cm) and *Aureobasidium pullulans* (1-2 cm). Viability of host parenchyma was easily visualized by TTC immersion. Non-infected sapwood turned a deep red colour (viable parenchyma) whereas heartwood and infected sapwood did not. *C. coerulescens* grew more rapidly in fresh logs than other species. This species typically caused a deep radial stain, often reaching the heartwood boundary, while other species such as *Ophiostoma* spp. only penetrated a short radial distance (Fleet et al., 2001).

Nutrition consumption. For the examination of nutrition consumption by sapstaining fungi, logs and sawnwood were infected with 14 fungal species belonging to *Ophiostoma, Leptographium*, and *Ceratocystis* genera. Both in infected billets and sawnwood, mannose was overall the most depleted mono-sugar (99% depletion), followed by glucose (70% depletion). Meanwhile, no significant levels of starch-bound glucose were found both in control and in infected samples. For lipids, the content of triglycerides (TG) was more focused due to its importance in fungal nutrition. In inoculated logs, deep-stainers, *C. coerulescens* and *Leptographium,* reduced TG content to very low or undetectable amounts and increased the fatty acids (FA) and resin acids (RA) content by 4 to 9 mg g^{-1} OD wood. Infection with *O. piceae* and *O. minus* resulted in the least TG reduction. This might indicate that deep-stainers produce larger amounts or more robust extracellular lipases than the other fungi (Fleet et al., 2001).

Pigmentation. To understand the properties of stain color caused by sapstaining fungi, significant pigmentation was analytically measured in the infected samples versus non-infected control wood. *C. coerulescens* infected wood was considerably more pigmented than the *Leptographium* spp. stain area. *Leptographium* isolates typically showed a non-pigmented, dead host cell zone approximately two to five cm ahead of the stained area within the logs. The kill zone of *Leptographium* was not substantially darker than the non-infected control wood. *Leptographium* mycelia were isolated from this area shortly after cutting and conidiophores grew in the same zone after two days of post-cut incubation. *C. coerulescens* and *Ophiostoma* spp. had much smaller dead-host zones (0 to 0.5 cm). However, none of the fungi required living host tissue for growth and pigmentation as demonstrated by their growth and pigmentation on g-sterilized wood.

Further tests were done to know the degree of pigmentation of *Leptographium, O. piliferum, C. coerulescens*, and *O. piceae* on defined media amended with different carbon

sources. Mannose consistently yielded the densest growth and the dark color in all the tested fungi. *Leptographium* and *O. piliferum* had identical color scoring on all the carbon sources. *O. piceae* had a very similar coloring except for glucose and linoleic acid. Interestingly, *C. coerulescens* has an almost revered ranking order compared to other species. Linoleic acid yielded dense growth and dark brown color in *C. coerulescens* had negligible growth on glycerol. However, this carbon source yielded good growth and dark pigmentation in the other fungi. These results suggested that carbon source is one of factors affecting pigmentation in sapstaining fungi (Fleet et al., 2001).

Finding of pigment production pathway

Based on the reports of earlier works that several darkly pigmented ascomycete fungi utilize dihydroxynaphtalene (DHN) melanin pathway (Fig. 3) and some of genes encoding enzymes involved in DHN melanin biosynthetic steps are known, pigmentation study in sapstain fungi targeted melanin genes. Consequently, scytalone dehydratases (SD), THN reductase (THNR), and pentaketide synthase (PKS) gene fragments were amplified from *O. floccosum* 387N strain by PCR with degenerate primers. Genomic clones containing a full length of the three genes were isolated from an *O. floccosum* lamda genomic library using the PCR-amplified DNA fragments as probes and the three genes' sequences were determined. The molecular works confirmed that the cloned *O. floccosum* genes are melanin genes encoding SD, PKS, and THNR based on their high sequence similarity with other fungal SD or THNR genes and their ability to restore melanin production in SD- or THN- deficient mutants of *Colletotrichum* or *Magnaporthe* fungi, respectively (Eagen et al., 2001; Wang and Breuil, 2002; Wang et al., 2001). These works demonstrated sapstain fungi use dihydroxynaphtalene (DHN) melanin pathway for their pigment production. Extended analysis with more numbers of sapstain species conformed that sapstain fungi use DHN melanin pathway (Fleet and Breuil, 2002).

Fig. 3. An example of the effect of Cartapip treatment on pine log protection from sapstain. Left: Cartapip-treated log. Right: untreated log, Sapstain is shown in the Cartapip-untreated log.

Sapstain control using albino technology

Test of albino strain. To apply the use of melanin information on the control of problematic sapstaining fungi, a potential biocontrol agent Cartapip™, an *O. piliferum* albino strain has been tested by Adnan Uzunovic (Forintek Canada Corp.). In a field trial in a sawmill in Alberta, Canada, the treatment of albino strain protected lodgepole pine logs from sapstain (Fig. 3). This trial involved 6-weeks-incubation periods.

Developing monitoring makers for biocontrol strain. The success of field trial led to develop makers that tracking the applied Cartapip in fields. For this, molecular techniques were used with wood samples (a total of 48 samples, 12 samples/disk) from a sawmill in Alberta where Cartapip sprayed for wood protection test. It was successfully to detect the presence of Cartapip in lodgepole pine logs disks using PCR-RFLP by *Hae*III and PCR assay with Cat1-Cat2 primers. It was also demonstrated that the Cat2-Cat2 primers were useful for detecting Cartapip in Germany where Cartapip was sprayed for controlling sapstain in scots pine lumber and logs (Schroeder et al., 2000, 2001a).

Since rRNA- or β-tubulin gene-based molecular markers for detecting Cartapip has limitation in use at some geographical regions (Schroeder et al., 2001b). Lee et al. (2002) assessed the feasibility of transforming with the green fluorescent protein (GFP), the sapstain fungus *Ophiostoma piceae* and a potential biocontrol agent Cartapip™, an *O. piliferum* albino strain. Transformants of the two fungal species were screened by PCR and Southern blot analyses. The GFP was expressed in spores, synnemata, and mycelia of the transformants grown in artificial media or wood (Fig. 4). The growth, pigmentation, and wood colonization of the GFP-transformants were similar to that of the non-transformants, suggesting that the

Fig. 4. Confocal microscopic image of green fluorescent mycelia of a GFP transformant of *Ophiostoma piliferum* albino strain in wood parenchyma cells.

presence of the *gfp* gene have no negative effect on the biology of the transformants. Using fluorescence and confocal microscopy, the GFP-expressing fungi were easily detected and differentiated from the wild type strains and other fungal species in wood, even four months after inoculation (Lee et al., 2002).

Outlook

Information on the biology of *Ophiostoma* fungi and their colonization on conifer has been increased continuously during last few years. Currently researches on the finding of genes involved in *Ophiostoma*-pine interactions, EST analysis of carbon metabolic genes of *O.* piceae and *O. novo-ulmi*, and analysis of *O. piliferum* genome are underway in the laboratories of Colette Breuil (University of British Columbia, Canada), Seong Hwan Kim (Dankook, University, Korea), Louis Bernier (Laval University, Canada) and investigators with the Centre for Structural and Functional Genomics at Concordia University, the Pulp and Paper Research Institute of Canada, the Institut national de la recherche scientifique - Institute Armand-Frappier in Laval and Pointe Claire, and the National Research Council Canada - Biotechnology Research Institute in Montreal. In the near future, these approaches will surely broaden our knowledge on *Ophiostoma* fungi and eventually, led us to further explore the relationship between *Ophiostoma* fungi and their hosts, and to use them in biotechnological applications.

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