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Frequency of Blue Staining Fungi isolated from Pine Trees of Experimental Forests in Kangwon National University and Its Resistance to Fungicide, Woodguard*1

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ABSTRACT

This study was performed to investigate the frequency of blue staining fungal species collected from pine trees, Experimental Forests of Kangwon National University in Korea based on their morphological characteristics. In addition the tolerance to fungicide, Woodguard, was assessed to get basic knowledges for preventing blue stain of wood.

Totally *Leptographium*-type fungi were dominated by 79.3% among Ophiostomatoid fungi associated with scolityd bark beetles in pine trees. *Leptographium*-type Ds-isolates which have unusual morphology were collected as frequency of 17.0%. The most distinct differeneces of these Ds-isolates from *L. procerum* were the presence of roughened hyphae and flask-shaped conidiophores that have never been mentioned formerly for *L. procerum*, but since these Ds-isolates formed black concentric rings being a property of *L. procerum*, the Df-isolates were characterized as *Leptographium*-type fungi, which are the most common species with the highest frequency by 33.2% in this particular area. According to our experimental results, *Leptographium*-type Ds- and Df-isolates were very resistant to fungicide, Woodguard, therefore it was suggested that a new method for wood protection from the blue staining fungi should be developed. Exact identification of blue staining isolates collected from pine trees is keep going.

Keywords : blue staining fungi, ophiostomatoid fungi, *Leptographium*-isolates, Ds-isolates, fungicide, Woodguard

1. INTRODUCTION

Blue staining fungi belonging to *Ophiostoma, Ceratocystis, Ceratocystiopsis,* and *Leptographium* genera are well known to be transferred by bark-beetles and wood-boring insects. These vectors provide delivery of blue staining fungi into vascular tissues of weakened trees or freshly cut logs that are the most suitable substrate for inhabiting the both partners. This phenomenon

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is of great practical importance because of economic losses that are results of wilt diseases and stain of sapwood caused by the fungi in trees and logs (Paine *et al.*, 1997, Jacobs and Wingfield, 2001). Studying blue staining fungi and their vectors is necessary for control of these associations and prevention of losses in wood industry. A recent report has provided strong evidence that blue staining fungi occur with high frequency (more than 90%) in population of bark beetles from pine trees (Lee *et al.*, 2003).

In order to understand biological properties of the blue staining fungi isolated from pine trees, Experimental Forests of Kangwon National University in Korea and to obtain basic knowledge of preventing discoloration by fungicide treatment, this study pursued morphological characteristics of collected blue staining fungal species and preliminary investigation of tolerance to fungicide, Woodguard.

2. MATERIALS AND METHODS

2.1. Study Site and Trap Log Preparation

Collection of bark beetles using trap logs of *Pinus rigida, P. rigitaeda* and *P. koraiensis* was done in the Experimental Forests of Kangwon National University, Hongchon, Kangwon-do. Ten trap logs were cut from 15- to 20-year-old pine trees into 70 cm length, and stacked in the forest stands of each pine tree.

2.2. Fungal Isolation from Collected Bark Beetles

Trap logs were collected every week from the end of March to the early July. Galleries in the bark and on the cambium were carefully exposed by peeling bark. Over-wintered adults, eggs and larvae were collected with sterilized forcep. Those adults, eggs, larvae and galleries were placed on the surface of blue staining fungi selective media (20 g Difco malt extract, 20 g agar, 0.1 g cycloheximide, 0.1 g streptomycin sulfate, 1 liter distilled water), and the plates were incubated at 20°C in the dark for 14 days. Actively growing mycelia were transferred to 2% malt extract agar (MEA) medium, and the pure cultures were kept at 4°C for further experiments.

2.3. Morphological Features of Blue Staining Fungi

To isolate separate fungal colonies, sterilized 2% MEA was added with lactic acid (4 ㎖/1000 ㎖ MEA). For every culture small amounts of inoculum (fungal conidia) were placed in centrum of 3～5 agar plates by inoculating needle. Petri dishes with plates were sealed with parafilm and incubated at 25°C from 2 weeks to 2 months. Long cultivation was used to check on perithecium forming. To obtain separate colonies the standard spread plate technique was used. For microscopy, relevant structures were mounted in lactophenol with 0.1% aniline blue or in lactic acid with 0.1% fuchsin acid on glass slides. From 10 to 20 measurements of each relevant structure were made in every culture. The all features obtained for the similar type isolates were listed. Cultural-morphological characteristics of the isolates were compared with literature data (Hunt, 1956; Jacobs and Wingfield, 2001).

2.4. Tolerance of the Isolates to Fungicide

In order to evaluate the tolerance of the isolates to fungicide, Woodguard from Hanchem Chemical Co., Korea, was used. This fungicide

consists to main component of 15% active ingredient (a.i.) of 3-iodo-2-propynyl-5-carbonyl compounds (IPBC) (Hanchem Chemical Co., Korea). Three isolates (*Ophiostoma*-type Lbm 46, *Leptographium*-type Ds 181 and *Leptographium*-type Df 229) were selected, since they represented three types of fungal cultures that were shown the most fast and intensive sapstain in pine woods according to our observations. One more *Ophiostoma minus* culture (M 5) isolated from pine tree (*Pinus sylvestris*) in Siberia (Russia) was added for the comparison. Agar plates of 2% MEA were inoculated with conidia suspensions of these four cultures, and incubated for 14 days to obtain mycelial mats. Every fungal isolate was grown in 10 agar plates of 100 mm in diameter. pine sapwood was used to produce small patterns, approximately $5\sim6\times7\times1.5\sim2$ mm. The patterns were dipped for 30 min in 200 ㎖ water solution of fungicide with concentration of total ingredient as follows: 0.06, 0.12, 0.25, 0.50, and 0.5%. Control patterns were treated with 0.015% $KMnO_4$ solution. In each variant of treatment the total amounts of sapwood patterns were 400, and raw weights of sapwood varied from 12 to 15 g. After treatment the patterns were soaked up with filtering paper and placed in Petri dishes on surface of mycelial mat. Each Petri dish contained 60 patterns spaced in 6 rows: 10p. control set, 5×10p. experimental sets. As 10 Petri dishes with culture of the same isolate were prepared, the amount of sapwood designed for testing each isolates equaled 600 patterns (100p. control and 5×100p. experimental set). The total amounts of sapwood patterns used in the experiment reached 2,400. Petri dishes with patterns were incubated 10 days at 25°C, then sapwood was transferred to moisture chambers (40 glass Petri dishes with filtering paper) keeping the primary order of pattern distribution inside Petri dishes. The whole incubation was continued for 3 weeks including 10 days on mycelial mat surface and 11 days in moisture chambers. At 21 days the patterns with signs of blue stain were counted without measuring intensity of blue stain. Probability method (Belenkov, 1991) was used to assess the tolerance of the isolates to fungicide by percentages of stained patterns. Additionally, probit-analysis was carried out to compare toxic effect of the fungicide on the isolates studied. In this case, percentage of non-stained patterns was presented as probability units (probits) that are normalized deviates $[t=(X-Xi)/\sigma]$ added by 5 to get rid of negatives (Belenkov, 1991). For every isolate relationship between probits and decimal logarithms of fungicide concentrations was approximated to linear graph. These graphs were used for determination of fungicide concentrations that had to provide 95% probability of wood protection (Belenkov, 1991).

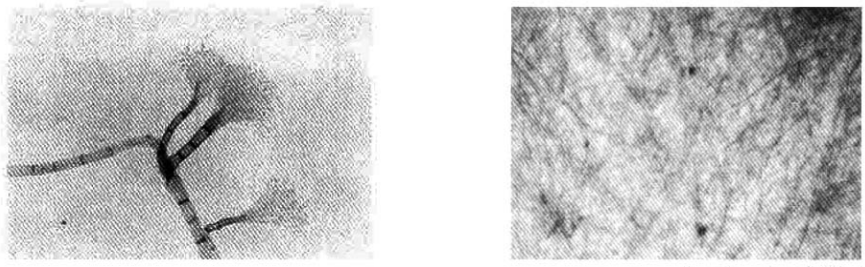
Table 1. Frequency of Ohiostomatoid fungi among 135 isolates from pine trees

Cultural type	Frequency (%)
Leptographium-type Df isolates	33.2
Graphium-type Gy isolates	21.1
Leptographium-type Ds isolates	17.0
Ophiostoma-type Lbm isolates	14.1
Ophiostoma-type Gw isolates	12.6
Leptographium-type Dfl isolates	7.4
Leptographium-type Le isolate	0.6

3. RESULTS and DISCUSSION

3.1. Frequency of Blue Staining Fungi

One hundred and thirty five blue staining fungi were isolated from pine trees of the experimental forests. Based on morphological properties, seven morphological types were set off among of dark-pigmented isolates as shown in Table 1. Isolates of four types formed *Leptographium*-type conidiophores, and key charac-



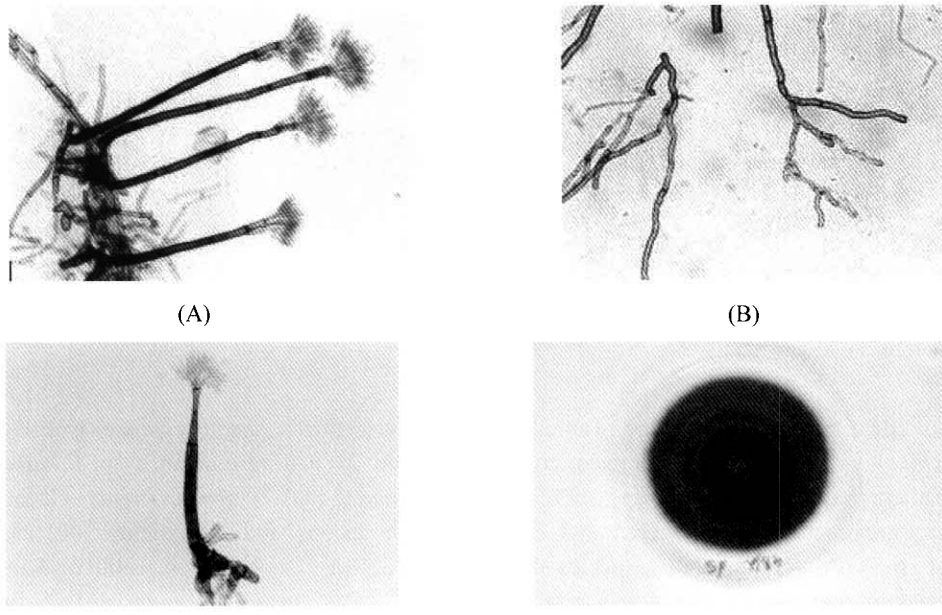
(A) (B)

Fig. 1. Mycological and cultural characteristics of Df-type isolates. (A) relatively short conidiophores, (B) black sclerotia in agar.

teristics of them are listed in the Table 2. *Leptographium*-type cultures were the most common with their frequency reaching up to 79.3%. Two *Ophiostoma* sp. (Lbm and Gw) and one *Graphium* sp. (Gy) were the other species that we observed in this study.

Leptographium-type Df isolates were the most common among other *Leptographium*-type cultures as frequency of 33.2% (Table 1). Comparison of morphological characteristics of this type with known *Leptographium* showed a vague similarity between Df isolates and *L. pineti* and *L. pinidensiflora. L. yunnanensis, Ophiostoma crassivaginata* and *O. robustum* were considered for their short conidiophores (Fig. 1A) (Jacobs *et al.*, 2000; Jacobs and Wingfield, 2001; Jacobs *et al.*, 2001a; Jacobs *et al.*, 2001b; Kim *et al.*, 2004). No one of these known species was in good correspondence with characteristics of these Df isolates. Moreover, such properties of Korean isolates as black sclerotia in agar (Fig. 1B) and coiled aerial hyphae were never mentioned in description of *Leptographium* species (Jacobs and Wingfield, 2001). For this reason these Df-type isolates need further investigation. The same thing could be shown about Dfl-type of fungal isolates (Table 1 and Table 2); *L. euphes, L. pineti* and *L. pinidensiflora* characteristics (Jacobs and Wingfield, 2001) were similar, but not identical to these Dfl-type isolates.

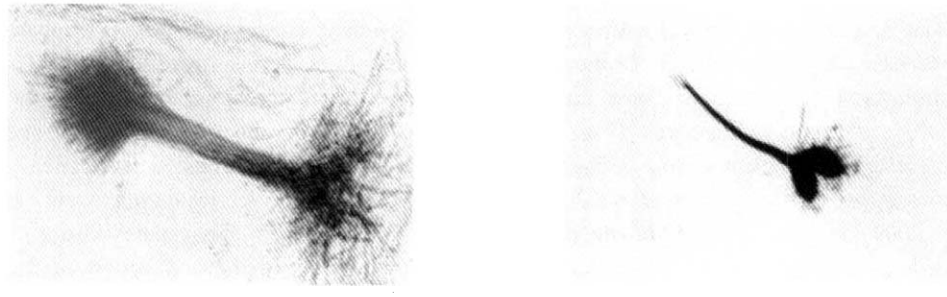
According to sizes of conidiophores, conidia and growth rate, *Leptographium*-type Ds isolates (Table 2) had resemblance to *L. albopini, L. lundbergii* and *L. neomexicanum* (Jacobs *et al.*, 2000; Jacobs and Wingfield, 2001; Jacobs *et al.*, 2001a; Jacobs *et al.*, 2001b; Kim *et al.*, 2004). But there was some distint differences among these three species and the Ds-isolates; *L. albopini* has up to 6 primary branches and does not form rhizoids-like structures that we observed in Ds-isolates (Fig. 2A). Rhizoids are also absent in *L. lundbergii* cultures. Unlike DS-isolates, *L. neomexicanum* is characterized by abundant aerial mycelium and C-type of primary branches: more than two, with a single large branch in the middle (Jacobs and Wingfield, 2001). The most coincidence between Ds-isolates and *L. procerum* were observed. Characteristics of Ds-isolates were in good correspondence with early report of the fungus (Kendrick, 1962), but they differed from the modern description in some measurements (Jacobs and Wingfield, 2001). The most distinct differences of Ds-isolates from *L. procerum* were the presence of roughened hyphae (Fig. 2B) and flask-shaped conidiophores (Fig. 2C) that have never been mentioned formerly for *L. procerum* (Jacobs and Wingfield, 2001). On PDA culture, these Ds-isolates formed black concentric rings (Fig. 2D) being a property of *L. procerum*. Therefore, attribution of Ds-isolates to *L. procerum* species needs more detailed investi-



(A) (B)

(C) (D)

Fig. 2. Mycological characteristics of Ds-type isolates. (A) rhizoid-like structures at the bottom of conidiophores, (B) rhoughened hyphae, (C) flask-shaped conidiophore, (D) black concentric rings in the culture colony. (by clockwise direction).



(A) (B)

Fig. 3. Mycological characteristics of Gw-type isolates. (A) synnematous *Graphium*-like conidiophore, (B) perithecium with neck, ostiolar hyphae, and empty base.

gation. As it is known that *L. procerum* is widespread throughout the world, *L. procerum* associated with many vectors and has a lots of host-plants from *Pinaceae* family (Jacobs *et al.*, 2000; Jacobs and Wingfield, 2001; Jacobs *et al.*, 2001a). It suggests that high probability of *L. procerum* presents in Korea. However, this fungus was not found in Japan in spite of serious investigation of blue staining fungi (Jacobs and Wingfield, 2001).

Isolates of Gw-types (Table 1) were characterized by synnematous conidiophores as they produced *Graphium*-like conidiophores with distinct stalk and head (Fig. 3A). Isolates of Gw-type formed *Sporothrix* like conidiophores together with synnematous ones. Three perithecia

(A) (B)

Fig. 4. Mycological characteristics of Lbm-type isolates. (A) perithecia with short neck and released ascospore mass, (B) crescent-shaped ascospores.

with empty bases (Fig. 3B) were found in culture of Gw-type, than showing Gw-cultures might be a *Ophiostoma sp.* (Table 1).

Seventh type of isolates (Lbm) formed small perithecia and black sclerotia in cultures. On 2% MEA medium, the colonies showed white color at first, with sparse aerial mycelium, gradually turned to deep-brown or black. Perithecia superficial to submerged, the bases black, globose, 60～100 μm in diameter; neck black, short, often bent, 40～100 μm long, 8～13 μm in diameter at the tip, 15～25 μm at the base; osiolar hyphae sometimes lacking, when present hyaline, blunt at the tips, variable in number, 8～25×1～1.5 μm; ascospores crescent-shaped, 4～5×1～1.5 μm. Conidiophores simple, tapering, 5～30×2～3 μm, sometimes branched, forming a brush-like mass. Conidia hyaline, aseptate, oblong with truncate bases and rounded apices, 2～8×1～2 m. Numerous black sclerotia develop on the surface of the agar, globose or irregularly-shaped, up to 1 mm in diameter, sometimes with imbedded peritecia. Growth rate of the colonies was about 40 mm in diameter in 5 days at 25°C on 2% MEA. According to morphological characteristics, this species would be considered to *Ophiostoma* sp. (Wingfield *et al.*, 1999; Harrington *et al.*, 2001; Kim *et al.*, 2003), and needs further exact identification. Gw-type isolates formed *Sporothrix* like conidiophores together with synnematous ones. Three perithecia with empty bases were found in culture of Gw-type, that showing Gw-isolates might be *Ophiostoma* sp. (Table 1).

The single culture of *Leptographium*-type Le isolates was isolated from *Hylurgops interstitials* gallery in *Pinus rigitaeda.* Though conidiophores and conidiogenous apparatus of this isolate were shorter, morphological characteristics of these Le isolates (Table 2) demonstrated similarity to known species *Leptographium elegans* M.J. Wiengf., Crous and Tzean. Though this was reported as species combining both *Leptographium*- and *Sporothrix*-conidiophores (Jacobs and Wingfield, 2001), lately *Leptographium bistatum* having *Sporothrix* synanamorph from *Pinus radiata* has been isolated (Lee *et al.*, 2003; Kim *et al.*, 2004). Our results expanded to the region of *L. elegans* distribution, as previously this species was reported from Taiwan only with *Chamaecyparis formosensis* as its host-plant (Jacobs *et al.*, 2000; Jacobs and Wingfield, 2001; Jacobs *et al.*, 2001a; Jacobs *et al.*, 2001b).

3.2. Resistence to Fungicide, Woodguard

It was proposed to assess tentatively a range of fungicide concentrations as a comparatively rapid laboratory technique that could provide a high level of wood protection in field trials

Table 2. Cultural and morphological characteristics of Leptographium like fungi associated with blue stain in pine trees

Characteristics	Df-type	Dfl- type	DS-type	Le-type
Length of conidiophores, μm	40~175	50~250	70~900	90~190
Length of conidiogenus apparatus, μm	40~175	20~100	20~130	15~40
Width of stipes at upper and basal part of conidiophores, μm	2~5 2~5	2~4 3~7.5	2~5 3~12.5	2~5 3~6
Primary branches: amount and type of branching	1~3, type B	2~3, type B	2~3 (4), type B	2~3, type B
Sharp and size of conidia, μm	Oblong, 3~6(8)×1~2(3)	Oblong, 3~6(7)×1~2(3)	Oblong, 3~5×1~2	Oblong, 3~5(6)×1
Rhizoid-like structures	absent	present	present	absent
Roughened hyphae	present	present	present	absent
Width of hyphae, μm	2~10(20)	2~12	2~6	2~6
Aerial mycelium	From middle to abundant at the edge of Petri dish	little	little	From little to middle
Diameter of colony at 2% MEA at 25°C, mm	55 (4 days) 79 (5 days)	46 (4 days) 62 (5 days)	31 (5 days) 39 (6 days)	8 (5 days) 15 (11 days)
Additional characteristics	Flat strands of 2~6 thick parallel hyphae on the agar surface. Spiral coiled aerial hyphae. Black sclerotia inside and on the surface of agar plates, 60~230 μm in diameter.	-	Black clubs of rhizoids. Black and white concentric rings in colonies on PDA. Swollen basal part of conidiophores in some isolate (flask-shaped conidiophores)	*Sporothrix*- like synanamorph. Hyphae aggregated in strands.

(Belenkov, 1991). Probit analysis is a common procedure in toxicology. Representative isolates of these three types and one *O. minus* culture isolated from pine tree in Siberia were used for the comparison. As shown in Table 3, there were differences in tolerance to the fungicide, Woodguard, between *O. minus* and *Leptographium*-type isolates. *O. minus* growth was completely inhibited in wood pattern treated with 0.50% of fungicide. The isolate Ds 181 did not colonized wood treated with 0.75% (Jacobs and Wingfield, 2001) of the fungicide, whereas Df 229 infected more than 20% of wood pattern after the same treatment. Df- and Ds-cultures equally as *O. minus* isolates caused the most fast and intensive staining of pine logs.

Probit graphs of the Woodguard toxic effect on blue staining fungi (Fig. 5) enabled to de-

Table 3. Influence of the fungicide, Woodguard

Fungal isolates	Percentage of stained wood patterns after incubation (21 days, 25°C) Concentration of fungicide in water solution (% total a.i.) 0.0	0.06	0.12	0.25	0.50	0.75	Equation of linear probit-graph (based on probit-graphs, Fig. 1)	Concentration of the fungicide providing 95% probability of protection from blue stain, % (based on probit-graphs, Fig. 5)
M 5	100	29.5	13.3	4.8	0.0	0.0	y = 2.2641x + 8.5739	0.14
Lbm 46	100	15.0	3.9	2.9	0.0	0.0	y = 2.5774x + 8.5500	0.18
Ds 181	100	83.5	67.0	43.3	9.6	1.0	y = 2.8745x + 7.2928	0.6
Df 229	100	100	76.7	69.9	29.1	21.9	y = 3.1954x + 6.4463	1.16

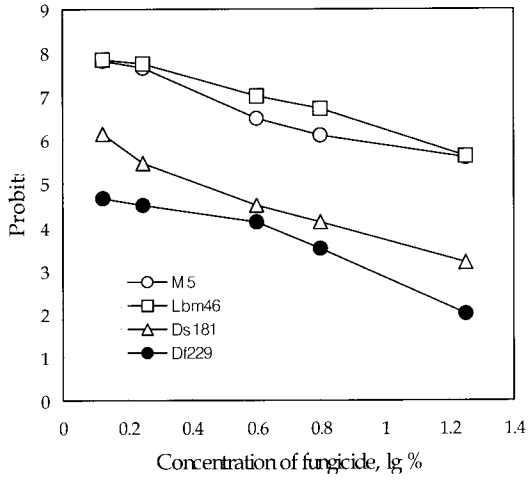


Fig. 5. Toxic effect of the fungicide "Woodguard" on blue staining fungi.

termine concentrations of the fungicide that had theoretically to provide 95% probability of protection of wood from colonization with isolates that were studied. As in Table 3, in the case of two *O. minus* isolates, this concentration is about 0.2% total a.i., and it amounts to 0.6 and 1.16% total a.i. for Ds 181 and Df 229, respectively.

The results were compared to the experimental data from recent report by Korean scientists recently (Song *et al.*, 2003). These authors tested different fungicides on their ability to inhibit fungal stain of Korean pine wood in field condition. It was reported that stain rating in pine boards treated with Woodguard solutions containing 0.75 and 1.0% total a.i. (active ingredient), which did not exceed of 19.8 and 8.0, respectively (Song *et al.*, 2003). Despite of differences in techniques and criteria used for assessment of blue stain expansion, the results of our laboratory experiment characterizing Df-type of *Leptographium*-like cultures are in agreement with the results of field trials (Song *et al.*, 2003), because in the both cases the most probability of wood protection (about 100%) occurred at Woodguard concentration about 1% total a.i..

4. CONCLUSION

Leptographium-type fungi were dominated by 79.3% among the ophiostomatoid fungi associated with scolityd bark beetles in pine trees, Experimental Forests of Kangwon National University in Korea. The most distinct differeneces of *Leptographium* Ds-isolates from *L. procerum* were the presence of roughened hyphae and flask-shaped conidiophores. These Ds-isolates formed black concentric rings being a property of *L. procerum*, and are also characterized as the most common species with high frequency by 33.2%. *Leptographium*-type Ds-isolates which have unusual morphology were collected as frequency of 17.0%. According to our experi-

mental results, since these Ds- and Df-isolates are very resistant to fungicide, Woodguard, therefore a new method should be applied for discolorization of wood against to blue staining fungi. Exact identification of blue staining isolates collected from pine trees is keep going.

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