

Biocontrol of Blue Stain in Pine Wood with Lyophilized Mycelium of *Ophiostoma quercus* Albino Strain

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Mycelium of *Ophiostoma quercus* albino strain cultured in liquid culture media was harvested, lyophilized, and stored for examining biocontrol efficacy against wood discoloration by staining fungi in the laboratory and field conditions. Dry weight of mycelium grown in brown sugar yeast extract broth (BYB) showed 3.8 times higher than that grown in potato dextrose broth (PDB). The optimum culture period in BYB was 4 weeks. In vitality test of the albino strain, the lyophilized mycelium stored in liquid nitrogen (−196°C) or in a refrigerator (4°C) kept the vitality until 13 months after storage; however, the mycelium stored at room temperature lost the vitality completely after 13 months. The mycelium stored in liquid nitrogen or in a refrigerator protected wood chips from the discoloration by pre-treating mycelial suspension on pine wood chips. The mycelium stored at room temperature for 7 months also showed complete protection. These results suggest that the lyophilized mycelium have a biocontrol efficacy only if it keeps the least vitality. In the field conditions, both albino strain and Woodguard[®] (commercial chemical protectant) showed significant differences ($p=0.05$) in discoloration rate as compared to the non-treated control when these were treated on the wood logs of *Pinus rigida*. The albino strain showed better protection than Woodguard[®]. Isolation frequency of blue stain fungi from the chips of wood logs treated with the albino strain was 0% at three months after treatment, while that treated with Woodguard[®] was 76.7%. In another experiment, pre-treatment of mycelial suspension on the cut surface of wood logs also showed significant protection from wood discoloration. Spraying of both albino strain on the cut surface and insecticides on the bark also showed relatively good control effects as compared to insecticide alone on the bark or non-treated control.

Keywords : biocontrol, blue stain fungi, Cartapip[®], *Ophiostoma minus*, *O. piliferum*, *O. quercus* albino strain, wild type strain, Woodguard[®]

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Wood is one of the essential and natural materials that is widely exploited for the various purposes including road and building constructions, furniture, paper production, and so on. Through all processes of wood production, wood decay and stain are the major limiting factors causing decrease of commercial value of wood products (Agrios, 1997; Basham, 1970; Liese and Peek, 1984). Wood stains mainly occur as wood discoloration by stain fungi, decay fungi, or chemical reaction during wood dry processes, but the most common case is caused by stain fungi (Alexopoulos et al., 1996; Gibbs, 1993; Zabel and Morrell, 1992).

Wood stain fungi cause wood discoloration in blue to dark and lowering the quality of wood, but is known not to affect on wood structure and tensility (Chapman and Scheffer, 1940; Seifert, 1993). Discolored wood chips need much more chemical agent for bleaching during mechanical pulping process, and subsequently accelerate environmental pollution (Behrendt and Blanchette, 1997; Seifert, 1993).

Wood stain fungi, usually called blue stain fungi, including *Ophiostoma*, *Ceratocystis*, *Leptographium*, and *Sphaeropsis*, first penetrate into wood through cut surface, and grow in the sapwood by disintegrating and absorbing chemical compositors of woods, like carbohydrates, fatty acids, triglycerides, and others (Harrington et al., 1998; Robinson, 1962; Rumbold, 1941; Wang et al., 1995; Wingfield and Gibbs, 1991; Wingfield et al., 1993; Jacobs and Wingfield, 2001). Wood stain fungi synthesize the pigment, melanin, in hyphae (Zimmerman et al., 1993; Zink and Fengel, 1988) and wood parts invaded by mycelium appear discolored by mycelial growth and expansion through parenchyma cells (Scheffer, 1973).

For the effective protection of woods from the attack of wood stain fungi, active researches have been carried out in USA (Behrendt et al., 1995a; Behrendt et al., 1995b; Benko, 1989; Blanchette et al., 1992; Farrel et al., 1992; Farrel et al., 1993), Europe (Chakravarty et al., 1994; Tarocinski and Zielinski, 1982), New Zealand (Farrel et al., 1998), and Japan, but rarely done in Korea (Pashenova et al., 2005). We reported the potentials for biological control of blue stain on wood caused by ophiostomatoid fungi in 2000 (Lee and Oh, 2000). Here, we report biocontrol efficacy of

Ophiostoma quercus albino strain using different storage methods and periods against wood discoloration in the laboratory and field conditions.

Materials and Methods

Mycelial growth of *Ophiostoma quercus* albino strain.

Ophiostoma quercus albino strain, BSFcs-1, was grown on Potato Dextrose Agar (PDA) plates at $23\pm 2^\circ\text{C}$ in the dark for 5 days, and then agar discs were cut off from the growing edge of colony using a cork borer (6.0 mm diameter). Agar discs were inoculated and shake-cultured in various liquid culture media (Table 1) at $23\pm 2^\circ\text{C}$ in the dark with 120 rpm for 2 weeks. Chemical compositions of culture media per liter of water were as follows; PDB: 24 g Potato Dextrose Broth (Difco), BSB: 30 g brown sugar: BMB: 30 g brown sugar and 3 g malt extract, BMP: 30 g brown sugar, 3 g malt extract, 0.5 g KH_2PO_4 and 0.5 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, BYB: 30 g brown sugar and 3 g yeast extract, BYP: 30 g brown sugar, 3 g yeast extract, 0.5 g KH_2PO_4 and 0.5 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$.

Mycelia were harvested by filtering through cheesecloth and on chromatography paper (Whatman®; 3 mm Chr) in a Baermann funnel, and lyophilized in a freezer dryer (Ilshin FD5505P), and then the dry weights were measured and compared. One gram of lyophilized mycelium stored in a refrigerator was diluted with 100 ml of sterile water, and 2 ml of the mycelial suspension was inoculated in 250 ml BYB. The growing mycelium was harvested and lyophilized every 2 weeks during incubation at $23\pm 2^\circ\text{C}$ in the dark with 120 rpm for 6 weeks. The weight of mycelium was measured as described above.

Lyophilization and storage. Agar discs taken from *O. quercus* colony on PDA were inoculated in 250 ml BYB (brown sugar 30%+yeast extract 3%), incubated at $23\pm 2^\circ\text{C}$ in the dark with 120 rpm for 25 days, and then mycelium was harvested. Harvested mycelium with the sterilized 10% skimmed milk in test tube was lyophilized and stored up to 15 months at three different conditions, i.e., in liquid nitrogen (-196°C), in a refrigerator (4°C), and at room temperature ($23\pm 2^\circ\text{C}$).

Biocontrol trial in the laboratory conditions. Lyophilized mycelia of *O. quercus* albino strain stored in a refrigerator were compared in the maintenance of vitality by storage periods. One gram of stored mycelium was taken out and diluted with, 1,000 ml of sterilized water at 0, 1, 9, and 15 months after storage, respectively. Mycelial suspensions were sprayed on PDA plate, incubated at $23\pm 2^\circ\text{C}$ in the dark for 3 days, and then vitality was compared by examining mycelial growth. Mycelial suspension of each treatment

was spray-inoculated on 10 g of sterilized wood chips of *Pinus densiflora* and *P. rigida* in glass Petri dishes. The treated plates were incubated at $23\pm 2^\circ\text{C}$ for 20 days, and the degree of discoloration by the wild type strain were rated as follows according to percentages of discolored area on wood chips. 0: 0(%), 1: 1-10(%), 2: 11-25(%), 3: 26-50(%), 4: 51-75(%), 5: 76-100(%).

One gram of stored mycelium was taken out and diluted with 1,000 ml of sterile water at 1, 7, and 13 months after storage, respectively. Mycelial suspensions were sprayed on PDA plate, incubated at $23\pm 2^\circ\text{C}$ in the dark for 3 days, and then vitality of mycelium was compared by checking mycelial growth. In order to compare biocontrol efficacy of the stored mycelium against blue stain at laboratory conditions, 5 treatments, i.e., spray of mycelial suspension of the albino strain, agar discs inoculation of wild type strain (*O. minus*), inoculation of the wild type strain one week after the treatment of the albino strain, simultaneous treatment of both the albino strain and the wild type strain, and treatment of albino strain one week after the inoculation of the wild type strain, were done on 10 g of sterilized wood chips of *P. densiflora* in glass Petri dishes. The treated plates were followed by the same procedures as mentioned above.

Biocontrol trial in the field conditions. Lyophilized mycelium of *O. quercus* albino strain stored at the refrigerator for 12 months was suspended with sterile water at the concentration of 10^{-3} . Wood logs of 15-20 year old *P. rigida* were prepared in pine forest, and each log was 40 cm in length and 10-15 cm in diameter. Cut surfaces of wood logs were spray-inoculated with various treatments; sterile water, mycelial suspension of *O. quercus* albino strain, mycelial suspension of *O. minus* (wild type strain) one week after *O. quercus* albino strain inoculation, and mycelial suspension of *O. minus* one week after Woodguard® (0.2%) treatment. Inoculated wood logs were placed in pine forest for 3 months. Wood discoloration was checked, and 30 wood chips (2×2 mm) for each treatment were taken from the split surface of sapwood every month. Wood chips were placed on the *Ophiostoma*-selective media (20 g Difco malt extract, 20 g agar, 0.1 g cycloheximide, 0.1 g streptomycin sulfate and 1 l distilled water), and incubated at $23\pm 2^\circ\text{C}$ in the dark for 5 days. Discoloration rate of sapwood and isolation frequencies of the wild type (*O. minus*) or the albino strain (*O. quercus*) were examined.

For the preparation of biocontrol agents against blue stain, *O. quercus* and *O. piliferum* albino strain (Cartapip®) were cultured on PDA plates at $23\pm 2^\circ\text{C}$ in the dark for 7 days. Agar discs taken from the growing edge using a cork borer (6.0 mm diameter) were static-cultured in 50 ml MEB (20 g Difco malt extract, 20 g glucose, 1 g peptone and 1 l

distilled water) at $23\pm 2^\circ\text{C}$ in the dark for 2 weeks. Mycelial suspension was made of harvested mycelium and sterile water, and used as a biocontrol agent. Chemical protectants or insecticides such as Woodguard[®] (0.2%, 0.4%), cypermethrin (0.13%), pyridaphenthion (0.1%), and anti-transpirant Cloud Cover[®] (10%) were also used to compare control efficacy against blue stain with the biocontrol agents. Treatments are categorized into 3 groups; 1) Distilled water, albino strains (*O. quercus* and *O. piliferum*) or chemical protectant (0.2 and 0.4% Woodguard[®]) on the cut surface, 2) Insecticides (cypermethrin and pyridaphenthion) or anti-transpirant (Cloud Cover[®]) on the bark, 3) albino strain (*O. quercus*) or Woodguard[®] (0.2%) on the cut surface and insecticides or anti-transpirant on the bark in all combinations. All treatments were done by spraying biological or chemical agents on wood logs (40 cm in length and 10-15 cm in diameter) of 15-20 year old *P. rigida* in pine forest. Wood logs were split longitudinally into 2 pieces at 3 months after treatment. Discoloration percentages on split surface were rated and compared. All experiments were done in three replications and statistical significances were analyzed by Duncan multiple range test at $P=0.05$.

Results and Discussion

Mycelial growth of *O. quercus* albino strain. Mycelial dry weights of *O. quercus* albino strain cultured in BYB and BYP at $25\pm 2^\circ\text{C}$, 120 rpm for 2 weeks were 3.8 and 3.7 times higher than that of the same strain cultured in PDB, respectively (Fig. 1). Yeast extract was more effective nutritionally in mycelial growth of *O. quercus* albino strain than malt extract.

Dry weight of harvested mycelium after shaking-culture in BYB was gradually increased until 2 weeks of incubation, slightly increased until 4 weeks, and slightly decreased thereafter (Fig. 2). Thus, the optimum culture period for culturing *O. quercus* albino strain in BYB is thought to be four weeks.

Biocontrol efficacy on wood chips in the laboratory conditions. When mycelial suspensions of lyophilized mycelium of the albino strain stored at the refrigerator were spread and grown on PDA plate to compare the maintenance of vitality by storage periods, vitality was kept until 9 months after storage, but it was maintained at very low level after 15 months of storage. When mycelial suspensions of the lyophilized mycelium stored at a refrigerator for different periods were treated with wild type strain in combinations on sterilized wood chips of *P. densiflora* and *P. rigida* in glass Petri dishes, inoculation of the albino strain alone and pre-treatment of the albino strain before the

wild type strain completely protected wood chips from blue stain until 15 months after storage. Biocontrol against blue stain was completely obtained even if the vitality of lyophilized mycelium stored for 15 months was kept at a very low level. In contrast with pre-treatment of albino strain, simultaneous treatment of the albino and wild type strains showed fairly good protection just after lyophilization of mycelium, but showed no more biocontrol efficacy after one month of storage. Inoculation of the wild type strain only or pre-treatment of the wild type strain before the albino strain completely discolored wood chips into bluish black (Table 1). As a result, pre-treatment and colonization of albino strain before the attack of blue stain

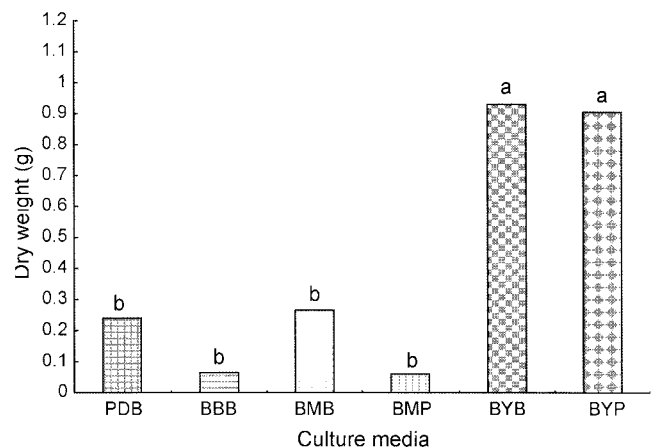


Fig. 1. Mycelial dry weights of *Ophiostoma quercus* albino strain cultured in various liquid media for 2 weeks ($25\pm 2^\circ\text{C}$, 120 rpm). The same letters on the bar denote no significantly different ($P=0.05$) by Duncan's multiple range test. PDB: potato dextrose broth (Difco), BSB: brown sugar, BMB: brown sugar+malt extract, BMP: Brown sugar+Malt extract+ KH_2PO_4 + $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, BYB: brown sugar+yeast extract, BYP: brown sugar+yeast extract+ KH_2PO_4 + $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$.

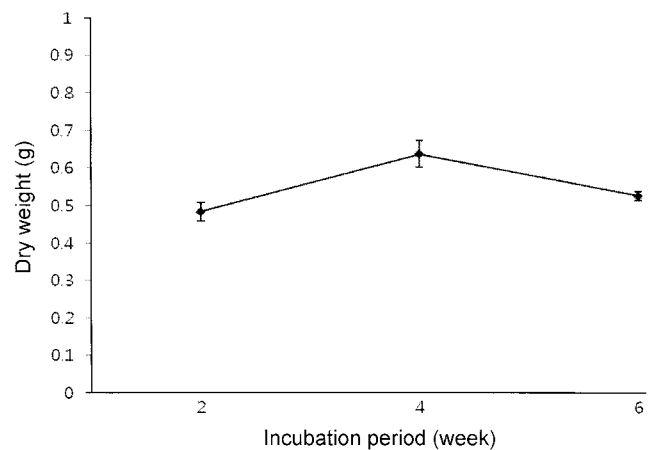
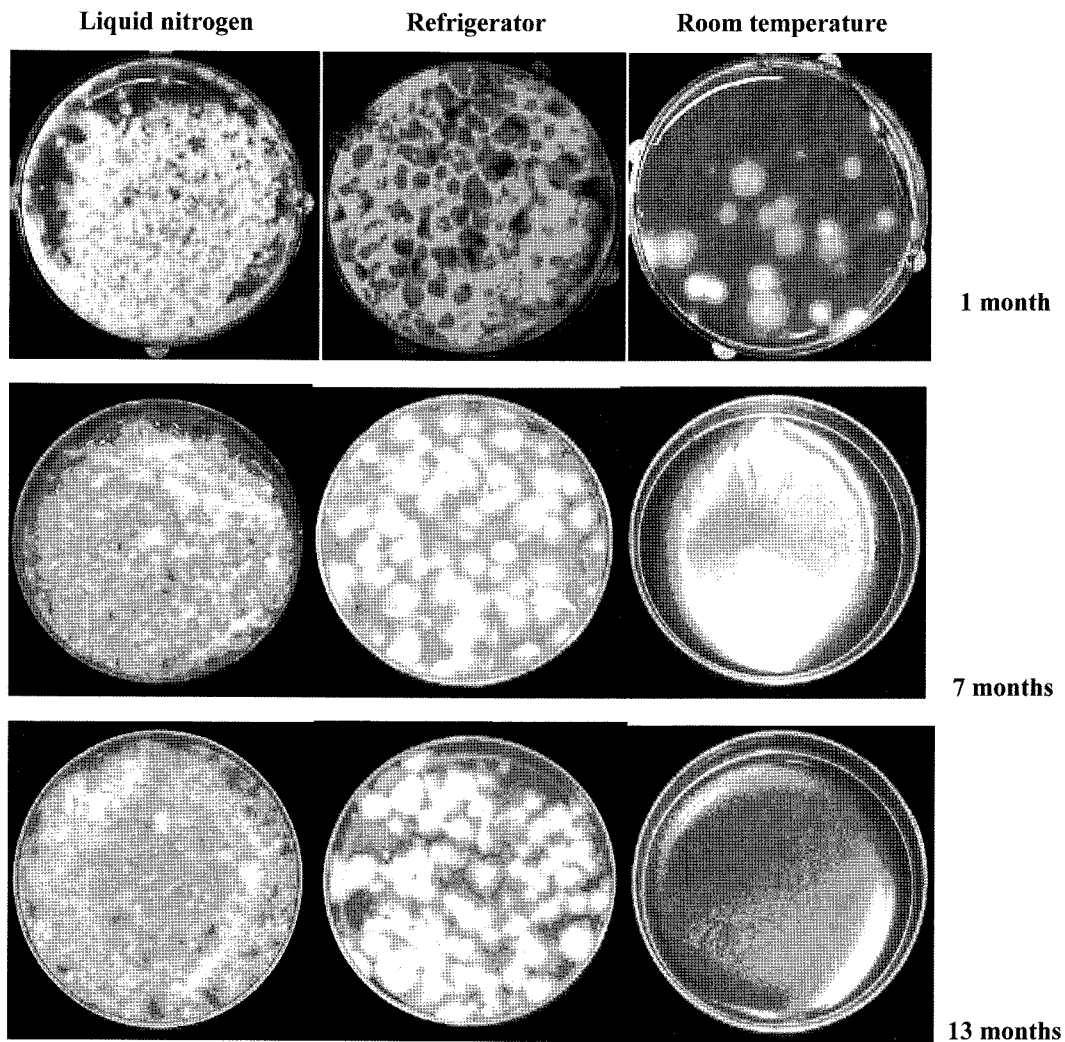


Fig. 2. Mycelial growth of *Ophiostoma quercus* albino strain, BSFcs-1, in BYB by incubation period. Error bar represents the standard deviation for the results of three replicates.

Table 1. Comparison of lyophilized and stored mycelium of *Ophiostoma quercus* albino strain in biocontrol against wood discoloration on wood chips of *Pinus densiflora* and *P. rigida*

| Tree species Storage periods (months) | <i>P. densiflora</i> | | | | <i>P. rigida</i> | | | |
|--|----------------------|---|---|----|------------------|---|---|----|
| | 0 | 1 | 9 | 15 | 0 | 1 | 9 | 15 |
| albino strain alone | 0* | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| wild type strain alone | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| wild type strain after albino strain | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| simultaneous treatment of albino and wild type strains | 0 | 4 | 5 | 5 | 1 | 4 | 5 | 5 |
| albino strain after wild type strain | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |

Albino strain: *Ophiostoma quercus* (BSFcS-1), Wild type strain: *O. minus*. *Discoloration rate of wood chips; 0: 0(%), 1: 1-10(%), 2: 11-25(%), 3: 26-50(%), 4: 51-75(%), 5: 76-100(%).

**Fig. 3.** Vitality of lyophilized mycelium of *Ophiostoma quercus* albino strain on PDA plate at 1, 7, 13 months after storage in liquid nitrogen, refrigerator, and room temperature, respectively.

fungi could protect wood chips of *P. densiflora* and *P. rigida* from blue stain, and biocontrol efficacy of lyophilized mycelium could be maintained until 15 months when these were stored at the refrigerator.

When mycelial suspensions of lyophilized mycelium were grown on PDA plates to compare vitality by storage conditions, vitality of lyophilized mycelium stored at room temperature ($23 \pm 2^\circ\text{C}$) was low after one month of storage,

very low after 7 months, and completely lost after 13 months. In case of the storing the lyophilized mycelium in a refrigerator (4°C), vitality was reduced after 7 months of storage as compared to that after one month of storage and there was no differences in growth activity between 7 months and 13 months of storage. However, vitality of lyophilized mycelium stored in liquid nitrogen (-196°C) was maintained up to 13 months of storage without any changes (Fig. 3). In conclusion, the best condition of lyophilized mycelium for keeping vitality at least for 13 months was the liquid nitrogen storage.

In biocontrol efficacy test of lyophilized mycelium stored at different storage conditions, where wood chips of *P. densiflora* were inoculated with the albino and wild type strains in combinations, wood chips treated with the wild type strain alone and pre-treatment of the wild type strain before the albino strain were severely blue-stained. On the other hand, inoculation of the albino strain alone and pre-treatment of the albino strain before the wild type strain completely protected wood chips from blue stain, except pre-treatments of the albino strain stored at room temperature for more than 7 months. It means that lyophilized mycelium cannot keep the vitality and biocontrol efficacy against blue stain for more than 7 months when it was

stored at room temperature. Simultaneous treatment of the albino and wild type strains showed variations in biocontrol efficacy among lyophilized mycelium of the albino strain stored at different conditions and periods. Lyophilized mycelium of the albino strain stored in liquid nitrogen showed slightly better efficacy than those stored at the refrigerator or room temperature (Table 2).

Biocontrol efficacy on wood logs in the field conditions.

Discoloration rates of *P. rigida* wood logs treated with different combinations in pine forest were checked every month for 3 months after treatments. One month later, discoloration rates by the treatments of sterile water, albino strain, wild type strain after albino strain, and wild type strain after Woodguard® were 1.0, 0.6, 0.6, 0.7%, and 8.3, 2, 2, 0.8% after 2 months, and 4.3, 2.3, 0.6, 3.8% after 3 months, respectively (Fig. 4). There were no significant differences among treatments after 1 month. However, after 2 months, all treatments except sterile water treatment showed efficient biocontrol results. However, results after 3 months showed that treatments of sterile water and Woodguard® were worse than the albino strain in preventing wood logs from blue stain. These results mean that it took at least more than one month for *P. rigida* wood logs

Table 2. Comparison of lyophilized mycelium of *Ophiostoma quercus* albino strain stored at liquid nitrogen, refrigerator or room temperature in biocontrol efficacy against wood discoloration on wood chips of *Pinus densiflora*

| Treatments | Storage methods | | | refrigerator | | | room temperature | | |
|--|------------------|---|----|-----------------|---|----|------------------|---|----|
| | periods (months) | | | liquid nitrogen | | | room temperature | | |
| | 1 | 7 | 13 | 1 | 7 | 13 | 1 | 7 | 13 |
| albino strain alone | 0* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| wild type strain alone | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| wild type strain after albino strain | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 4 |
| simultaneous treatment of albino and wild type strains | 1 | 3 | 5 | 2 | 5 | 5 | 5 | 5 | 5 |
| albino strain after wild type strain | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |

Albino strain: *Ophiostoma quercus* (BSFcs-1), Wild type strain: *O. minus*. *Discoloration rate of wood chips; 0: 0(%), 1: 1-10(%), 2: 11-25(%), 3: 26-50(%), 4: 51-75(%), 5: 76-100(%)

Table 3. Isolation frequencies of wild type and albino strains from wood chips of *Pinus rigida* wood logs placed in pine forest for 3 months after inoculation of the albino and wild type strains in combinations

| Treatments | Periods (months) | wild type strain | | | albino strain | | |
|--|------------------|------------------|-----------------|----------------|---------------|----------------|---|
| | | 1 | 2 | 3 | 1 | 2 | 3 |
| sterile water | 0/30 (0)* | 1/30 (3.3) | 1/30 (3.3) | 0/30 (0) | 0/30 (0) | 0/30 (0) | |
| albino strain alone | 0/30 (0) | 0/30 (0) | 3/30 (10) | 4/30 (13.3) | 15/30 (50) | 7/30 (23.3) | |
| wild type strain after albino strain | 3/30 (10) | 2/30 (6.7) | 0/30 (0) | 9/30 (30) | 9/30 (30) | 9/30 (30) | |
| wild type strain after Woodguard® (0.2%) | 3/30 (10) | 5/30 (16.7) | 23/30 (76.7) | 0/30 (0) | 0/30 (0) | 0/30 (0) | |

*The figures in parentheses represent mean percentages of isolation frequency. Albino strain: *Ophiostoma quercus* (BSFcs-1), Wild type strain: *O. minus*.

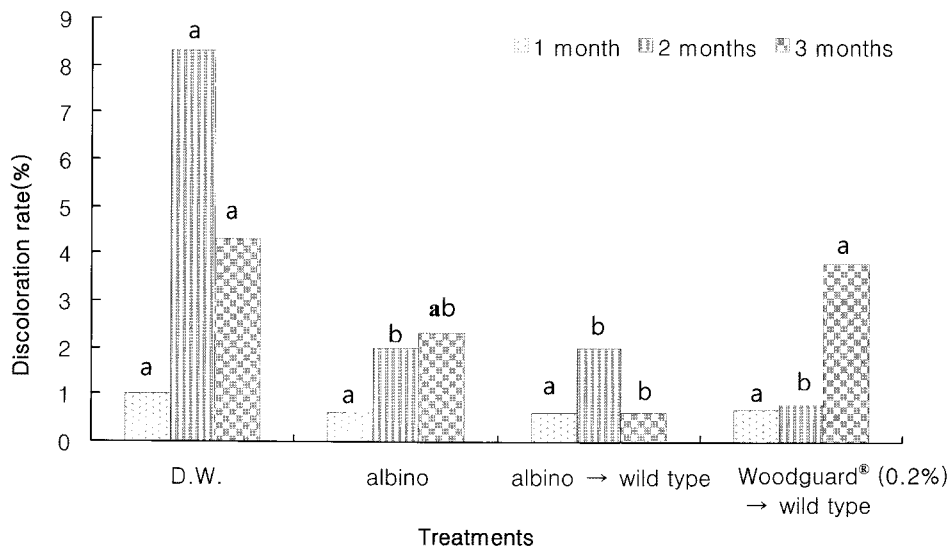


Fig. 4. Effects of various treatments on wood discoloration by incubation periods. The same letters above the bars in each treated period indicate no significant difference ($P=0.05$) by Duncan's multiple range test.

to be discolored by staining fungi in the forest, and Woodguard® could protect the wood logs from discoloration for 2 months, but the preventing effect was not persisting until 3 months after treatment in the field. After checking discoloration rate of wood logs, isolation frequencies of the wild type and/or albino strain from the treated wood logs were compared by placing and incubating 30 wood chips for each treatment on *Ophiostoma*-selective media. Isolation frequencies of the albino and wild type strains were 0, 0, 10%, and 13.3, 50, 23.3%, respec-

tively for 3 months in case of the albino strain treatment alone. In case of the wild type treatment after albino strain, those of the wild type and albino strains were 10, 6.7, 0% and 30, 30, 30%, respectively. On the other hand, in case of the wild type treatment after Woodguard®, isolation frequencies of the wild type strain were greatly increased as 10, 16.7, and 76.7% for 3 months, but no albino strain was isolated. In the treatment of sterile water, isolation frequencies of the wild type strain were 0, 3.3 and 3.3%, respectively, but the albino strain was never isolated (Table

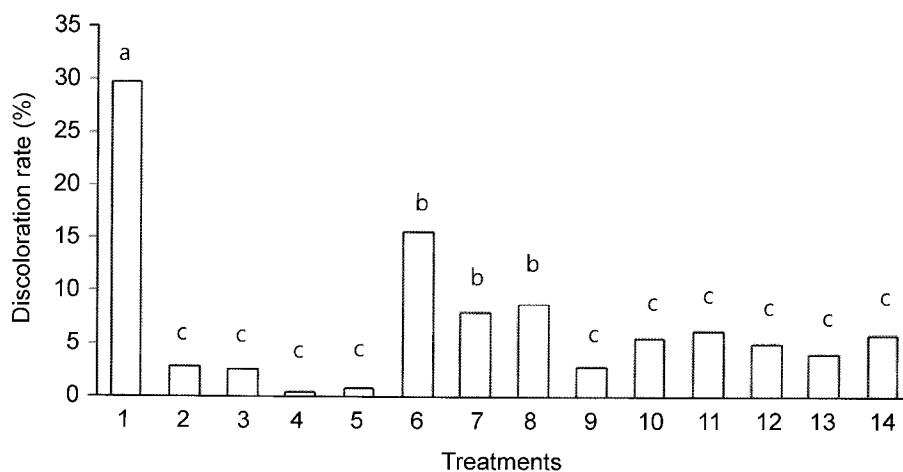


Fig. 5. Effect of albino strain and/or insecticides (cypermethrin and pyridaphenthion), anti-transpirant (Cloud Cover®), and wood protectant (Woodguard®) treatment alone or in combinations on the discoloration of wood logs in pine forest. 1-5) Distilled water, *O. quercus* albino strain, *O. piliferum* albino strain, 0.2% Woodguard® and 0.4% Woodguard® on the cut surface, respectively, 6-8) Cypermethrin, pyridaphenthion, and Cloud Cover on the bark, respectively, 9-14) *O. quercus* albino strain and cypermethrin, *O. quercus* albino strain and pyridaphenthion, *O. quercus* albino strain and Cloud Cover, Woodguard® and cypermethrin, Woodguard® and pyridaphenthion, and Woodguard® and Cloud Cover®, respectively, on the cut surface as well as on the bark. The same letters above the bars indicate no significant difference ($P=0.05$) by Duncan multiple range test.

3). Low isolation frequencies of the wild type strain despite of high discoloration rate in the treatment of sterile water may due to the use of *Ophiostoma*-selective media, which inhibit the growth of non-ophiostomatoid staining fungi.

Comparison of various treatments to prevent wood logs of *P. rigida* from wood discoloration showed that all treatments showed significant difference in discoloration rate of wood logs from treatment of sterile water as the control. Spray treatment of the albino strain or Woodguard® on the cut surface greatly decreased discoloration rates as compared to that by sterile water. Simultaneous treatments of insecticides (cypermethrin, pyridaphenthion) and anti-transpirant (Cloud Cover®) on the bark and the albino strain on the cut surface also showed satisfactory suppression of wood discoloration. However, treatments of insecticides or anti-transpirant alone on the bark did not give satisfactory prevention from wood discoloration (Fig. 5). These results indicate that the cut surface of wood logs is the primary attacking site of wood staining fungi, and insecticides or anti-transpirant play a role to repel bark beetles, which usually transmit ophiostomatoid fungi when they make holes to lay their eggs.

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