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Potentials for Biological Control of Blue Stain on Woods Caused by Ophiostomatoid Fungi

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Biological control of blue stain fungi, such as *Ophiostoma* and *Leptographium* spp., that reduce the quality of logs and cause economic losses in wood product industry, was carried out in laboratory and field trials by a colorless strain of *Ophiostoma quercus*, BSFcs-1. Inoculation of pine wood chips with the colorless strain 1 wk before inoculating wild-type strain demonstrated that BSFcs-1 colonized wood chips and excluded blue stain fungi from being established. Efficacy of BSFcs-1 was compared with a colorless strain of *O. piliferum*, which is commercially available under the trade name of Cartapip. Inoculation of pine wood logs with the colorless strain 1 wk before inoculating wild-type strain of blue stain fungi resulted in 50% colonization for the colorless strain in isolated wood chips, while *O. quercus* and *O. floccosum* colonized 0 % and 17%, respectively. Simultaneous inoculation of logs with the colorless and wild-type strains resulted in decreased colonization (28%) by BSFcs-1, but increased colonization by *O. quercus* (18%) and *O. floccosum* (29%). On the other hand, BSFcs-1 and wild- type strain alone colonized 75% and 71%, respectively. Treatment of the surface of log ends with mycelial suspension of BSFcs-1 after cutting also showed good control of blue stain fungi in a pine forest stands.

Keywords **:** biological control, blue stain, colorless strain, *Leptographium, Ophiostoma*, ophiostomatoid fungi

Blue stain fungi, mainly ophiostomatoid fungi such as *Ophiostoma* and *Ceratocystis* species, are one of the initial colonizers invading freshly cut wood and cause blue to black discolorations in sapwood (Fig. A). Fungal hyphae usually grow in ray parenchyma cells and resin canals (Fig. B) (Gibbs, 1993). Discoloration of sapwoods by fungal colonization is due to melanin, a pigment existing within the hyphae. Blue stain of wood greatly reduce the quality of timber and cause economic losses in wood products and paper industries (Seitfert, 1993).

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Control of blue stain fungi had been tried by dipping timber into water to inhibit fungal colonization, or application of fungicides. The most general and satisfactory process consists of dipping the freshly cut timber in a fungicide. In the past, the most generally used were ethyl mercuric chloride, ethyl mercuric phosphate, sodium pentachlorophenol, sodium tetrachlorophenol, and so on. Mercury-containing compounds are not used now because of hazards and the use of chlorinated phenols also has caused a major problem for the lumber industry. Thus, the need for developing other methods, which can replace the use of toxic chemicals, was required in the environmental aspects. Several investigations of biological control measures to control blue stain fungi were tried using several fungi or bacteria (Benko, 1988). Partially satisfactory results were obtained at the laboratory level, but not at the field level. In 1995, a non-pigmented strain of *Ophiostoma piliferum* was reported as an effective control agent for the inhibition of wild-type, pigmented blue stain fungi from colonization of freshly cut wood in the field. This strain was commercially developed in the trade name of Cartapip by Sandoz Chemical Co. (Behrendt et al., 1995a, b).

This study was carried out to evaluate the effectiveness of a melanin-deficient isolate (BSFcs-1) of *O. quercus* isolated from *P. rigida* in Korea for protecting cut wood from the colonization of wild-type, pigmented blue stain fungi, and to compare with commercially available Cartapip in the laboratory and field conditions.

Materials and Methods

Fungal isolates used. Blue stain fungi were isolated from the stained woods of *Pinus koraiensis, P. rigida* and *P. rigitaeda* collected in the experimental forest of Kangwon National University (KNU) located at Hongchon, Kangwon-Do, Korea. Among several different species of ophiostomatoid fungi, *Ophiostoma quercus* (Fig. C) and *Leptographium* sp. from *P. koraiensis*, and *O. floccosum* from *P. rigitaeda* were used in this experiment (Lee and Oh, 2000). For the control of blue stain fungi, a melanin-deficient isolate (BSFcs-1) of *O. quercus* isolated from *P. rigida* was obtained from the sector formed on the culture media (Fig. 1D). In order to compare the effectiveness of BSFcs-1 at protecting

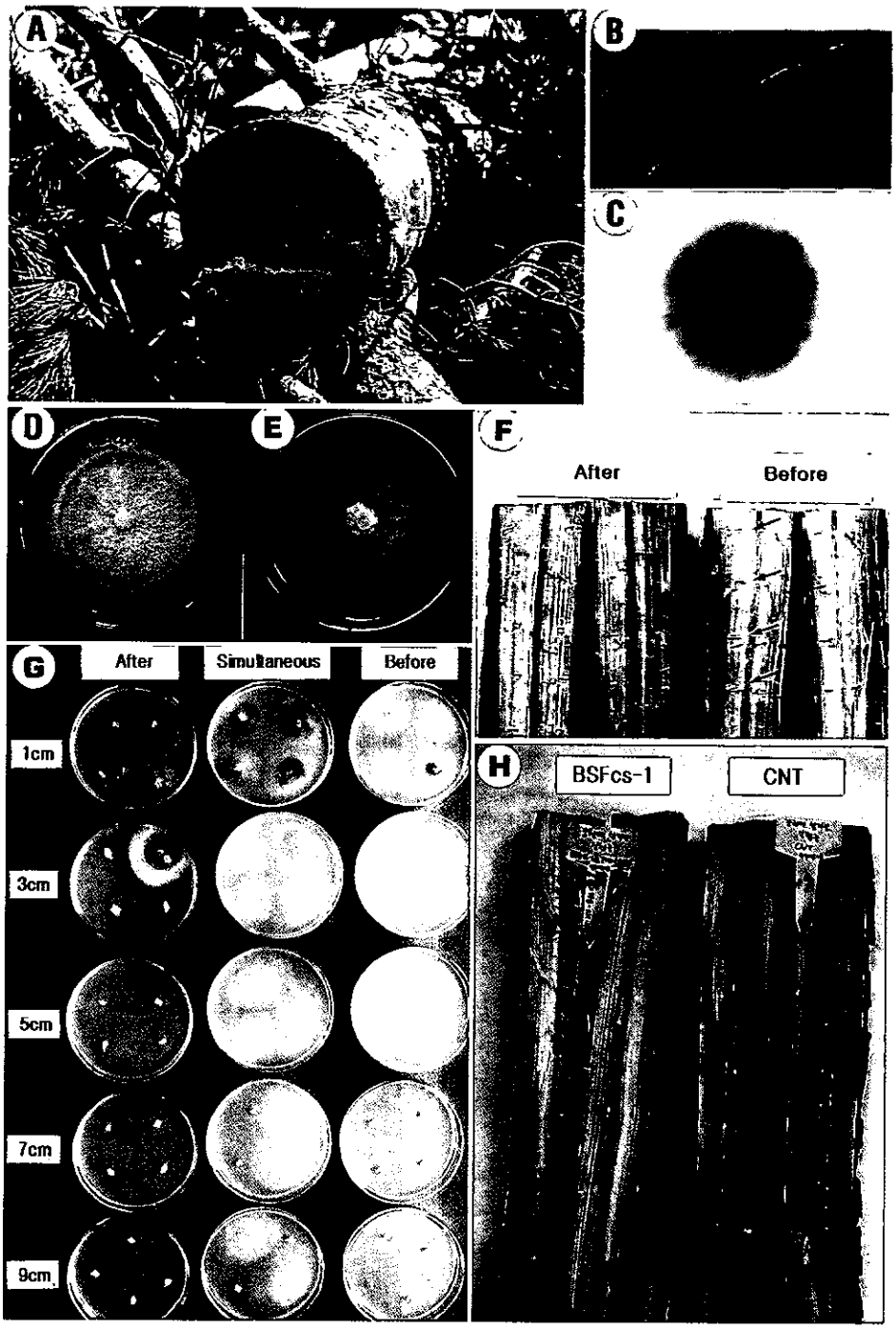

Fig. 1. A. Blue stain on freshly cut logs of *Pinus koraiensis* in the experimental forest of Kangwon National University (KNU) located at Hongchon, Kangwon-do, Korea. **B.** Typical sapstain on *Pinus rigitaeda* wood block. The blue stain on the cut surface of wood block is caused by *Ophiostoma floccosum*. **C.** Cultural morphology of *Ophiostoma quercus* isolated from *Pinus rigida* on 1.5% malt extract agar in the dark at 25±2°C for 14 days. Brown to dark brown color of mycelium is due to melanin, a pigment existing within the hyphae. **D.** Cultural morphology of BSFcs-1, a melanin-deficient and colorless strain of *Ophiostoma quercus* isolated from *Pinus rigida*. **E.** Cultural morphology of a colorless strain of *Ophiostoma piliferum*, which is commercially available by the name of Cartapip (Sandoz Chemical Corporation), and supplied by the Department of Plant Pathology, University of Minnesota. **F.** Wood logs of *Pinus rigida* treated with BSFcs-1 7 days after the inoculation of wild-type strain of *Ophiostoma* sp. on the one side of log end were stained in the sapwood by the colonization of these fungi, while no wood stain was observed in the split sapwood by the treatment of BSFcs-1 7 days before the inoculation of wild-type strains. Arrows point the positions of sapwood where wood chips were taken for re-isolating wild-type strain and/or a colorless strain. **G.** Re-isolation of wild-type strain and/or a colorless strain from the sapwood of logs, when a colorless strain was

inoculated after, simultaneously, and before wild-type strain in the laboratory. Wood chips were taken from the sapwood of split surface at 1, 3, 5, 7, 9 cm from the end of logs, and 4 chips with the same distance from the end of log were placed on *Ophiostoma* selective media. **H.** Biological control effects of BSFcs-1 by pre-treatment on the cut surface of the logs. left: mycelial suspension of BSFcs-1 was treated before piling logs in a pine forest stand, and no blue stain is detected on sapwood after 8 weeks; right: sterilized water was sprayed as the control, and sapwood was severely stained by blue stain fungi.

woods from wild-type blue stain fungi with Cartapip (Sandoz Chemical Corporation, Charlotte, N.C.), which is a colorless strain of *O. piliferum* and commercially available for the biological control of blue stain fungi, the colorless strain of *O. piliferum* was obtained from the Department of Plant Pathology, University of Minnesota, St. Paul, MN 55108, USA and kept in a deep freezer before use (Fig. E) (Blanchette, 1992; Farrell et al., 1993). All fungal isolates used were cultured on 1.5% MEA medium (15 g of Difco malt extract, 15 g of agar, 1000 ml of distilled water) in the dark at 25±2°C for 14 days.

Laboratory test on wood chips. In order to evaluate the effectiveness of the colorless isolate (BSFcs-1) on inhibiting wild-type, pigmented blue stain fungi from colonizing wood chips, 15 to 20 year old *P. densiflora* and *P. rigida* were felled and chipped at the experimental forest of KNU. The freshly cut wood chips were transported to the laboratory, and kept in plastic bags at -20 °C freezer. Wood chips (about 1.5 cm × 1.5 cm × 0.3 cm) in glass petri dishes were sterilzed at 121°C for 30 min., and then inoculated with agar plugs of both wild-type and colorless isolate of blue stain fungi. Agar plugs including mycelium were obtained from the margins of actively growing colony on the culture media by using sterilized cork borer (5 mm in diameter), and then placed up side down for direct contact of mycelium with wood chips. Treatments consisted of *O. quercus*, *Leptographium* sp., and *O. floccosum*, BSFcs-1 alone, BSFcs-1 inoculated simultaneously with each of the above fungi, BSFcs-1 inoculated 7 days before and after the inoculation of other fungi. Cartapip was treated exactly in the same way as BSFcs-1, and the effectiveness was compared with BSFcs-1. Wood chips of both *P. densiflora* and *P. rigida* were used as host substrates. A total of 46 plates were used with 3 petri dishes per treatment.

Laboratory test on wood logs. Fifteen to twenty year old *P. rigida* were felled. Stems and branches were cut into 30 cm sections and transported to the laboratory. Unsterilized fresh logs were inoculated within 2 days after cutting. For the protection of logs from the growth of saprophytic microorganisms, bark surface of sectioned logs were sprayed with 95% ethanol and flame-sterilized. One side of log end was wrapped with parafilm to protect from desiccation and contamination, and the other side was placed with the mycelial mat of wild-type and colorless isolate (BSFcs-1) of blue stain fungi grown in 1.5% MEB (15 g of Difco malt extract, 1000 ml of distilled water) in the dark at 25±2°C for 14 days. Fungal mat was evenly spread over the entire surface of log end using a sterile forceps, and covered with parafilm to prevent inoculum from desiccation. Simultaneous inoculation of BSFcs-1 and wild-type was done by placing both mats on a log end. Treatments were the same with the test on wood chips. Inoculated logs were placed on the moistened paper towel in plastic boxes (46 cm × 30 cm × 25 cm), and kept in the dark at 20±2°C for 12 weeks after inoculation.

Every week, the boxes were opened to change moistened paper towels for keeping moisture and to exchange the air in the box. Four, eight, and twelve weeks after inoculation, 3 wood logs per treatment were randomly sampled to investigate the extent of colonization of inoculated fungi by re-isolating these fungi again from the sapwood of logs. Sampled logs were flame-sterilized and split longitudinally into two equal parts with a sterile ax. Blue stain fungi were isolated by removing wood chips (about 2 mm × 2 mm × 1 mm) from the sapwood of the split surface. Wood chips were taken at 1, 3, 5, 7, 9 cm from the end of log, and 4 chips with the same distance from the end of log were placed on *Ophiostoma* selective media (20 g of Difco malt extract, 20 g of agar, 0.5 g of cycloheximide, 0.4 g of streptomycin sulfate, 1000 ml of distilled water) (Harrington, 1981). After 7-10 days, fungal colonies growing out from the wood chips on media were identified, and the percentage of colonization were determined. Mean percentage was determined by dividing the number of fungal colonies growing on media by the total number of wood chips removed from the log end to the distance of 7 cm into sapwood (average 16 chips).

Field test on wood logs. Field trials were carried out in the pine stands located at the campus of Kangwon National University in early April. Fifteen to twenty year old *P. rigida* were felled in experimental forest. Stems and branches were cut into 30 cm sections and moved to field test area. Logs were treated with mycelial suspension of the colorless isolate, and set aside 5 different sites.

Treatments consists of spraying of mycelial suspension of the colorless isolate (BSFcs-1) with hand sprayer at the both side of log end, and spraying of sterilized water without BSFcs-1 for the control. In order to prepare the biocontrol agent, BSFcs-1 of blue stain fungi was grown in 1.5% MEB in the dark at 25±2°C for 14 days. Mycelial mat was harvested and mycelial suspension was prepared by grinding mat with sterilized water added. Logs were collected 2 months after treatment, split into 2 pieces longitudinally, and the presence of wood stain was examined.

Results and Discussion

Laboratory observation of wood chips. Wood chips inoculated with *O. quercus*, *Leptographium* sp., or *O. floccosum* were stained into blue, gray, or black color by the colonization of these fungi, while those inoculated with BSFcs-1 or a colorless strain of *O. piliferum* alone were not stained at all despite of colonization by colorless isolates of blue stain fungi. Treatment with BSFcs-1 7 days before the inoculation of wild-type strains of blue stain fungi demonstrated no visible wood stains. Among wild-type strains inoculated, *Leptographium* sp. showed limited growth around the inoculated agar disc, while *O. quercus* and *O. floccosum* showed no growth (Table 1). These results mean that

Table 1. Comparison of BSFcs-1 and Cartapip in biological control effects on the sterilized wood chips of *Pinus rigida* and *P. densiflora*

Treatment[1)]	A						B						C						Wild-type strain only			BSFcs-1 only	Cartapip only
	BSFcs-1[a)]			Cartapip			BSFcs-1			Cartapip			BSFcs-1			Cartapip							
Host	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3[b)]		
Pinus rigida	−	+	−	−	+	−	+	+	+	+	+	+	++	++	++	++	++	++	++	++	++	−	−[c)]
Pinus densiflora	−	+	−	−	+	−	+	+	+	+	+	+	++	++	++	++	++	++	++	++	++	−	−

[1)] Treatment A: treatment of a colorless strain 7 days before the inoculation of wild-type strain; Treatment B: treatment of a colorless strain and wild-type strain simultaneously; Treatment C: treatment of a colorless strain 7 days after the inoculation of wild-type strain.
[a)] BSFcs-1: a colorless strain of *Ophiostoma quercus*; Cartapip: a colorless strain of *O. piliferum*.
[b)] Wild-type strains; 1: *O. quercus*; 2: *Leptographium* sp.; 3: *O. floccosum*.
[c)] Degree of blue stain on wood chips; +: light; ++: heavy; −: none.

BSFcs-1 pre-colonize on wood chips and exclude wild-type strains of blue stain fungi from being established. Simultaneous inoculation of BSFcs-1 and wild-type strains of blue stain fungi showed partial discoloration of wood chips. Inoculation of wild-type strains of blue stain fungi 7 days before the treatment of BSFcs-1 resulted in wood staining by the colonization of wild-type strains. The above results suggested that the successful control of blue stain fungi can be obtained by the pre-treatment of BSFcs-1 at least 7 days before the inoculation of wild-type strains of blue stain fungi. Both the colorless strain (Cartapip) of *O. piliferum* and the colorless strain (BSFcs-1) of *O. quercus* showed no visual differences in the effectiveness for the control of blue stain fungi. Generally, all strains used in this experiment showed better growth and sporulation on the wood chips of *P. rigida* than those of *P. densiflora*.

Laboratory observations of wood logs. Wood logs of *P. rigida* inoculated with wild-type strain of *O. quercus* and *O. floccosum* alone on the one side of log end were stained in the sapwood by the colonization of these fungi, while no wood stain was observed in the split sapwood by the treatment of BSFcs-1 7 days before the inoculation of wild-type strains (Fig. F). BSFcs-1 colonized 3 cm from the log end in 4 weeks, 7 cm in 8 weeks, and 9 cm in 12 weeks (Fig. G). The isolation percentage of BSFcs-1 from wood chips was ranged from 38 to 56%, while those of *O. quercus* and *O. floccosum* were 30% and 58%, respectively.

For *O. quercus*, treatment with BSFcs-1 7 days before the inoculation of wild-type strain resulted in no colonization of the wild-type strain but 19-31% isolation frequency of BSFcs-1, which means that the complete inhibition of blue stain fungi, can be obtained by pre-treatment of BSFcs-1 (Table 2).

For *O. floccosum*, the isolation percentage of wild-type strain was 50-63% when this strain was inoculated 7 days before BSFcs-1, 19-44% when both strains were simultaneously inoculated, and 0-27% when wild-type strain was inoculated after BSFcs-1 (Table 3). The wild-type strain was isolated from wood chips treated with BSFcs-1 after the inoculation of this fungus until 8 weeks, but this fungus was no more isolated at 12 weeks and isolation percentage of BSFcs-1 was up to 38%.

Generally, isolation percentage of wild-type strains was increased as the period of treatment increased when wild-

Table 2. Mean percentage of isolated wood chips from the sapwood colonized by wild-type (*Ophiostoma quercus*) and BSFcs-1 strains of blue stain fungi at different distances from the log end when BSFcs-1 was inoculated after, simultaneouly, or before wild-type in the laboratory

Periods after inoculation (wks)		4			8			12		
Treatments		A[1)]	B	C	A	B	C	A	B	C
Distance (cm)	1	75(0)[a)]	50(0)	0(75)	50(0)	67(33)	0(50)	100(0)	0(50)	0(100)
from log ends	3	−	25(0)	−	50(50)	50(50)	0(50)	25(0)	0(50)	0 (25)
	5	−	-	−	0(25)	0(25)	−	−	−	−
	7	−	25(0)	−	25(0)	0(25)	−	−	−	−
	9	−[b)]	−	−	−	−	−	−	−	−

[1)] Treatment A: inoculation of wild-type strain of blue stain fungi 7 days before BSFcs-1; Treatment B: simultaneous inoculation of wild-type and BSFcs-1; Treatment C: inoculation of wild-type strain 7 days after BSFcs-1.
[2)] Mean percentage was determined by dividing the number of fungal colonies growing on media by the total number of wood chips removed from the sapwood.
[a)] Each value and the figure in parenthesis represent the isolation percentage of wild-type strain and BSFcs-1, respectively.
[b)] −: no fungal isolation.

Table 3. Mean percentage of isolated wood chips from the sapwood colonized by wild-type (*Ophiostoma floccosum*) and BSFcs-1 strains of blue stain fungi at different distances from the log end when BSFcs-1 was inoculated after, simultaneouly, or before wild-type in the laboratory

Periods after inoculation (wks)		4			8			12		
Treatments		A[1)]	B	C	A	B	C	A	B	C
Distance (cm)	1	100(0)[a)]	100(0)	75(25)	100(0)	25(0)	75(0)	50(0)	75(25)	0(75)
from log ends	3	100(0)	0(100)	33(67)	100(0)	50(0)	0(75)	50(0)	0(100)	0(50)
	5	–	0(100)	–	25(0)	50(0)	25(25)	50(0)	0(100)	0(25)
	7	–	–	–	–	50(0)	–	100(0)	0(25)	–
	9	–[b)]	–	–	–	25(0)	–	50(0)	–	–

[1)]Treatment A: inoculation of wild-type strain of blue stain fungi 7 days before BSFcs-1; Treatment B: simultaneous inoculation of wild-type and BSFcs-1; Treatment C: inoculation of wild-type strain 7 days after BSFcs-1.
[2)]Mean percentage was determined by dividing the number of fungal colonies growing on media by the total number of wood chips removed from the sapwood.
[a)]Each value and the figure in parenthesis represent the isolation percentage of wild-type strain and BSFcs-1, respectively.
[b)]–: no fungal isolation.

type strain was inoculated 7 days before BSFcs-1. However, the percentage showed a reduction or no colonization as the period increased when wild-type strain was inoculated 7 days after BSFcs-1. The percentage was irregular when both strains were inoculated simultaneously. *O. floccosum* showed more rapid and deep colonization on the sapwood of *P. rigida* than *O. quercus* (Table 4).

Field observations of wood logs. When the wood logs treated with BSFcs-1 or sterilized water were harvested 2 months after the treatments, and the presence of wood stain was investigated on sapwood by splitting longitudinally into two equal parts, the wood logs treated with sterilized water without BSFcs-1 as the control showed complete staining by blue stain fungi, while no wood stain was detected by the treatment of the colorless strain (BSFcs-1) (Fig. H). These results indicate that the treatment of BSFcs-1 of *O. quercus* on freshly cut surface of wood can protect cut wood from the colonization of pigmented blue stain fungi in the field trials. These results also demonstrated that BSFcs-1 pre-colonized freshly cut sapwood and excluded blue stain fungi from being established. Through the laboratory tests on wood chips, wood logs, and the field test on wood logs, potentials for biological control of blue stain on woods caused by ophiostomatoid fungi were confirmed by the pre-treatment of the colorless strain of *O. quercus* (BSFcs-1). However, for the successful control of blue stains on wood, the colorless strain should have several characteristics, i.e., the ability of rapid and dominant colonization on the freshly cut wood logs before the establishment of other microorganisms in forest ecosystem, no conversion of the colorless characteristics into color by sexual reproduction with wild-type strains, and so on. Blue stain fungi can colonize the sapwood by the direct invasion of the both ends of cut logs as well as by the transmission of propagules into the sapwood after bark beetle attack. Thus, to maintain cut logs totally free from blue stain, it is required to control both fungal colonization and bark beetle attack. Future researches should be focused on the integrated control of blue stain fungi and insect vectors, bark beetles, in the field.

Table 4. Mean percentage of isolated wood chips from the sapwood colonized by *Ophiostoma quercus* or *O. floccosum* after 4, 8, 12 weeks when BSFcs-1 was inoculated after, simultaneouly, or before these fungi in the laboratory

blue stain fungi	*O. quercus*			*O. floccosum*		
	4	8	12	4	8	12 wks
post-inoculation	19Y	31Y	31Y	50Y	56YZ	63Z
simultaneously	25YZ	29Y	0X	25X	44Y	19Y
pre-inoculation	0X	0X	0X	27X	25X	0X

Mean percentage was determined by dividing the number of fungal colonies growing on media by the total number of wood chips removed from the log end to the distance of 7 cm into sapwood (average 48 chips).
Letters within a column are significantly different according to Fisher's LSD test (p=0.05).

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