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Pre-Infection Behavior of the Pitch Canker Fungus *Fusarium circinatum* on Pine Stems

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Pre-infection behavior of *Fusarium circinatum* on stems of pine species was investigated with scanning electron microscopy. Two-year-old stems of *Pinus densiflora* and *P. rigida* were inoculated with the fungal conidial suspension and subjected to 25°C for up to 16 hr. Most microconidia germinated 12 hr after inoculation on pine stems. Conidia produced germ tubes from either one or both ends of microconidia. Germ tubes grew over the stem surface and appeared to enter host tissues through natural openings on pine stems. Surface cracks in the cork were entrance sites of germ tubes of *F. circinatum*. In addition, host cell wall cracks were often found at the tip of germ tubes. The cuticle appeared to be eroded either at the tip of germ tubes or around germlings. Germ tubes also produced appressoria-like structures, exhibiting swollen tips of germ tubes on the stem surface. There seems to be no significant differences in the pre-infection behavior of *F. circinatum* on stems between the two pine species.

Keywords **:** appressoria, *Fusarium circinatum*, germination, *Gibberella circinata*, pine

Pitch canker is one of the most destructive diseases of pines worldwide. The disease was first described from the southeastern United States (Hepting and Roth, 1946). More recently, the disease has been reported in South Africa (Viljoem et al., 1994), Korea (Lee et al., 2000), and Chile (Wingfield et al., 2002). The disease is caused by an imperfect fungus *Fusarium circinatum* Nirenberg and O'Donnell (synonymous with *F. subglutinans* f. sp. *pini* and *F. moniliforme* var. *subglutinans*) (Correll et al., 1991; Nirenberg and O'Donnell, 1998). Its teleomorph is known to be *Gibberella circinata* Nirenberg and O'Donnell (Nirenberg and O'Donnell, 1998). The disease results in needle blight, dieback, and resin flows from the infected areas on the shoots, branches, cones, and trunk (Lee et al., 2000). Considerable variation in resistance to pitch canker was found among pine species. Pitch pine (*Pinus rigida* P. Mill.) and Japanese black pine (*P. thunbergii* Parl.) are known to be susceptible to pitch canker, whereas Japanese red pine (*P. densiflora* Sieb. & Zucc.) and Korean pine (*P. koraiensis* Sieb. & Zucc.) are resistant to the disease (Lee et al., 2000).

It is widely accepted that pre-infection behavior of plant pathogenic fungi is characterized by the sequential events of cellular differentiation (Kim et al., 1999 and references therein). The initial stage of infection includes the deposition and attachment of spores to aerial parts of the host plants. Spore germination leads to the subsequent formation of one or more germ tubes that direct their growth to infection courts. Many plant pathogenic fungi form infection structures like appressoria, which can be one of the most important events for host penetration and colonization (Hoch and Staples, 1987). The pitch canker fungus can infect through wounds caused by insect feeding, weather-related damages like wind and hail, or silvicultural practices (Gordon, 2006). In particular, wounds by beetles, weevils, and moths are known as a primary infection court of the fungal spores (Fox et al., 1991; Gordon et al., 2001; Lee et al., 2000). Insects contribute to the disease development and spread by either vectoring or the creation of optimal infection courts through their feeding activity (Schweigkofler et al., 2004). Disease management of pitch canker depends on careful stand management, minimizing pruning wounds and controlling insect vectors to reduce predisposition of hosts to infection by the pathogen (Barrows-Broaddus and Dwinell, 1984).

In order to unravel morphological details of the infection process of *F. circinatum* in pitch canker development, it is crucial to have a comprehensive understanding of the pre-infection behavior of the fungus on pine stems. Little information is available on the documentation of the fungal behavior on pine stems. The objectives of this study were i)

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to identify entrance sites of *F. circinatum* on pine stems and ii) to determine the infection process of the fungus in pitch canker development.

Materials and Methods

Plant materials. Two different pine species were used in this study: (i) *P. rigida* and (ii) *P. densiflora*. Two-years-old seedlings of each species were grown in a nursery at Suwon, Korea and were transferred to a growth chamber maintained at 25°C. The stems (approximately 10 mm in diameter) were cut into 10 mm lengths for inoculation.

Inoculum preparation. An isolate (FC-1) of *F. circinatum* was obtained from a naturally infected pine stem exhibiting typical pitch canker symptoms. The isolate formed micro- and macroconidia, polyphialides, and coiled hyphae (data not shown). The isolate was grown on potato sucrose agar (PSA) plates at 25°C under continuous fluorescent light with a 12-hr photoperiod for one week. The culture surface on the PSA plates was flooded with 10 ml of sterilized distilled water and scraped to make a conidial suspension. Inoculum concentration of the conidial suspension was adjusted to 1.0×10^4 microconidia/ml using a hemacytometer.

Inoculation of pine stems. Stem cuttings of each species were washed with mild detergent to remove dirt and air-dried. Inoculation sites were marked on stems using tweezers for point inoculation. A drop (50 μl) of the conidial suspension was placed on each inoculation site using an adjustable micropipette. Immediately after inoculation, the stems were placed in a petri dish lined with moist filter papers, and put into a polyethylene bag for maintaining wetness throughout treatment periods. They were placed in incubators at 25°C under continuous darkness. Following wetness periods of 2, 8, 12, and 16 hr, the stems were removed and subjected to specimen preparation for scanning electron microscopy to observe the fungal behavior on the stems.

Scanning electron microscopy. Squares of stems (each 5×5 mm^2 with approximately 1 mm thickness of underlying tissues) were excised using a razor blade from the stems. Uninoculated stems were also sampled and processed as a control. They were immersed in modified Karnovsky's fixative (Karnovsky, 1965) consisting of 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) at 4°C overnight, and washed with the same buffer three times each for 10 min. The specimens were postfixed with 1% (w/v) osmium tetroxide in the same buffer at 4°C for 2 hr, and washed briefly with distilled water twice. They were dehydrated in a graded ethanol series (30, 50, 70, 80, 95 and three times in 100%, each for 10 min) at room temperature. The specimens were further treated with isoamyl acetate twice each for 10 min, and dried in a critical point drier (CPD 030; BAL-TEC, Balzers, Liechtenstein) with liquid carbon dioxide as a transitional fluid. They were mounted on a metal stub (10 mm in diameter) using two-sided adhesive carbon tape and coated under an argon atmosphere with a thin layer (ap-

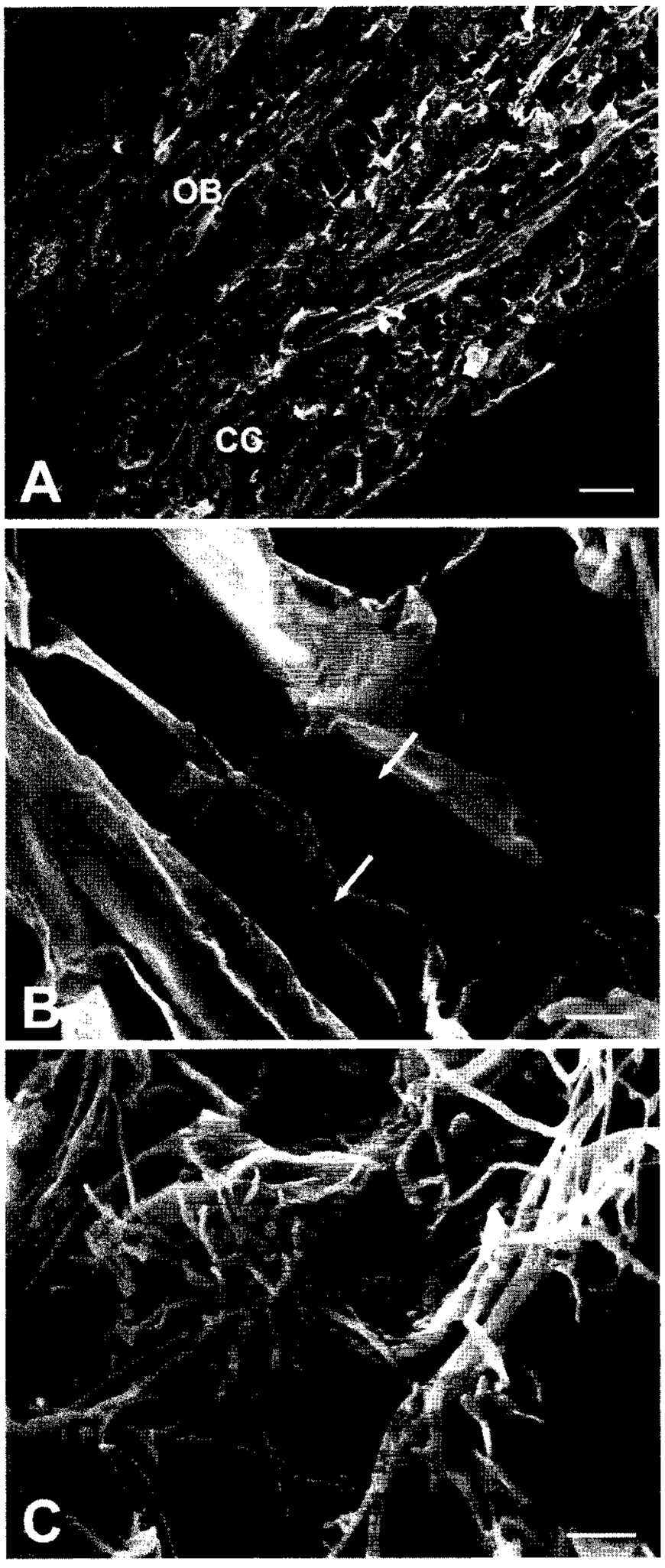

Fig. 1. Scanning electron micrographs of *F. circinatum* on stems of *P. rigida*. (A) Uninoculated outer bark (OB) surface and cork cells (CC). Bar=200 μm. (B) Ungerminated microconidia (arrows) on the cork cell surface 2 hr after inoculation. Bar=20 μm. (C) Conidial germination on the cork cell surface 12 hr after inoculation. Bar=20 μm.

proximately 30 nm in thickness) of gold using a sputter-coater (JFC-1100E; JEOL, Japan). The specimens were examined with a scanning electron microscope (JSM-5410LV; JEOL, Japan) at an accelerating voltage of 20 kV. Secondary electron images were recorded using a digital image processor (analySIS version 3.00; Soft Imaging System GmbH, Germany).

Results

Fungal behavior on stems of *P. rigida*. Scanning electron microscopy revealed uninoculated outer bark surface of *P. rigida* (Fig. 1A). Some of the outer bark cells were sloughed off, exposing the rough surface of cork cells. Ungerminated microconidia of *F. circinatum* were fusoid and straight in shape (Fig. 1B). Most conidia germinated 12 hr after inoculation and produced germ tubes from either one or both ends of microconidia (Fig. 1C). Germ tubes grew over the stem surface and appeared to enter host tissues through natural openings on pine stems (Fig. 2A). Higher magnifications clearly showed that seemingly surface cracks in the cork were entrance sites of germ tubes of *F. circinatum* (Fig. 2B). Instances were also noted where germ tubes seemed to either grow into indented areas or attempt to directly penetrate the host cell wall by perpendicular growth to the bark surface (Fig. 2C). In addition, host cell wall cracks were often found at the tip of germ tubes (Fig. 3A). The affected areas showed elongated cracks and were seemingly rather indented in higher magnifications (Fig. 3B).

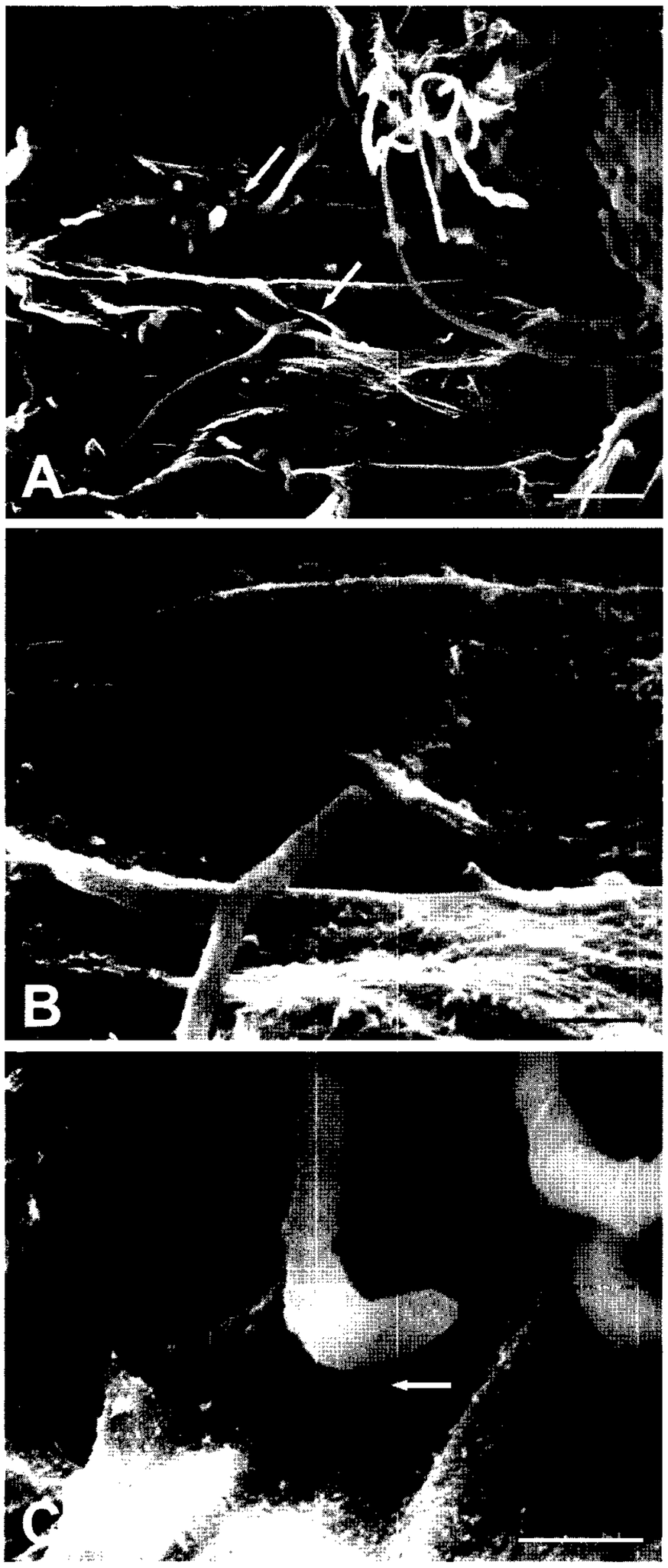

Fig. 2. Scanning electron micrographs of *F. circinatum* on stems of *P. rigida*. (A) Host entrance through cracks (arrows) by germ tubes 16 hr after inoculation. Bar=20 μm. (B) Higher magnification of host entrance. Bar=5 μm. (C) Hyphal growth on the cork cell surface 16 hr after inoculation. Note the perpendicular growth of a germ tube (an arrow) to the cork cell surface. Bar=5 μm.

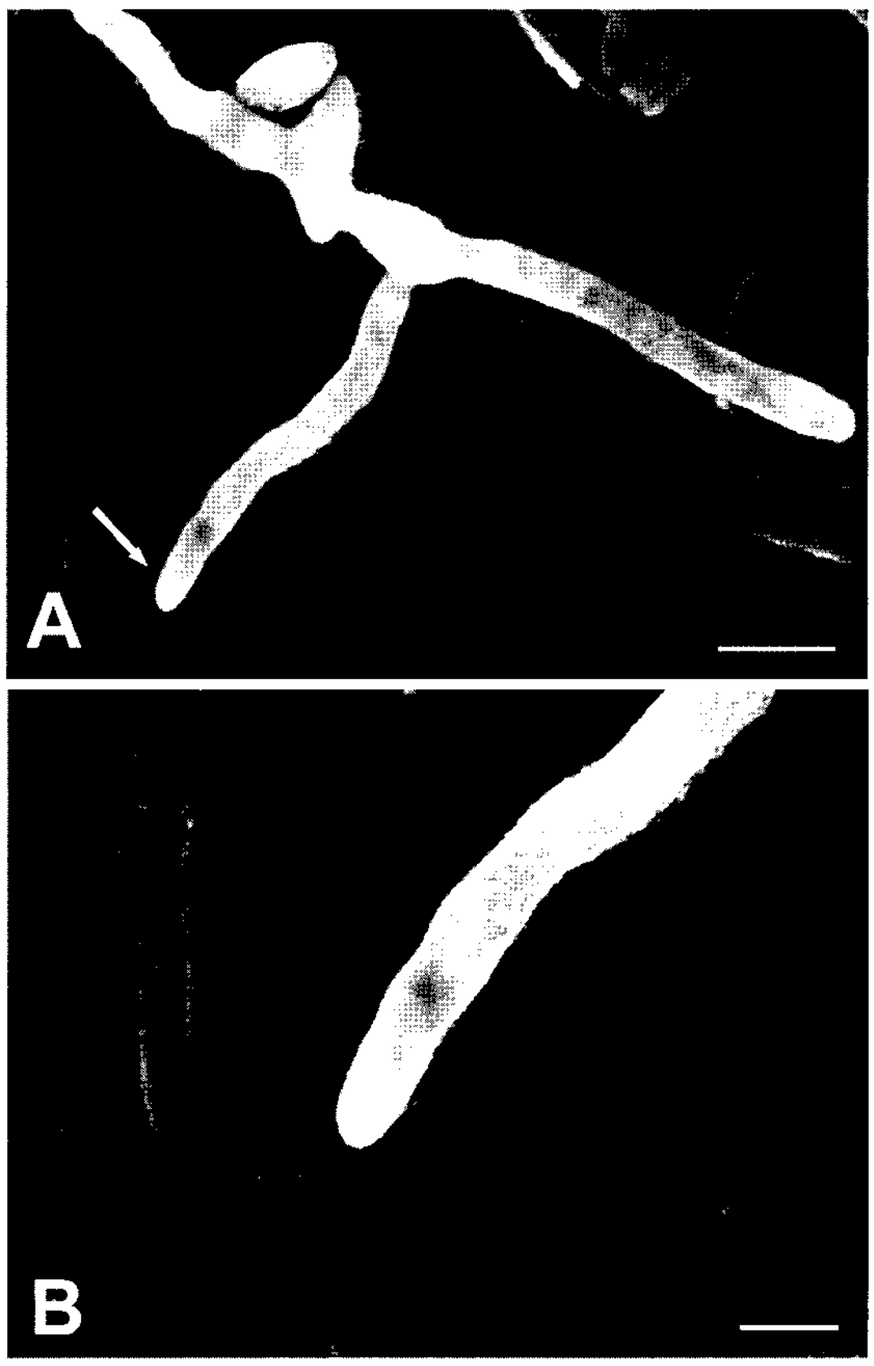

Fig. 3. Scanning electron micrographs of *F. circinatum* on stems of *P. rigida*. (A) Host cell wall crack (an arrow) at the tip of a germ tube 16 hr after inoculation. Bar=5 μm. (B) Higher magnification of the crack underneath the germ tube. Bar=2 μm.

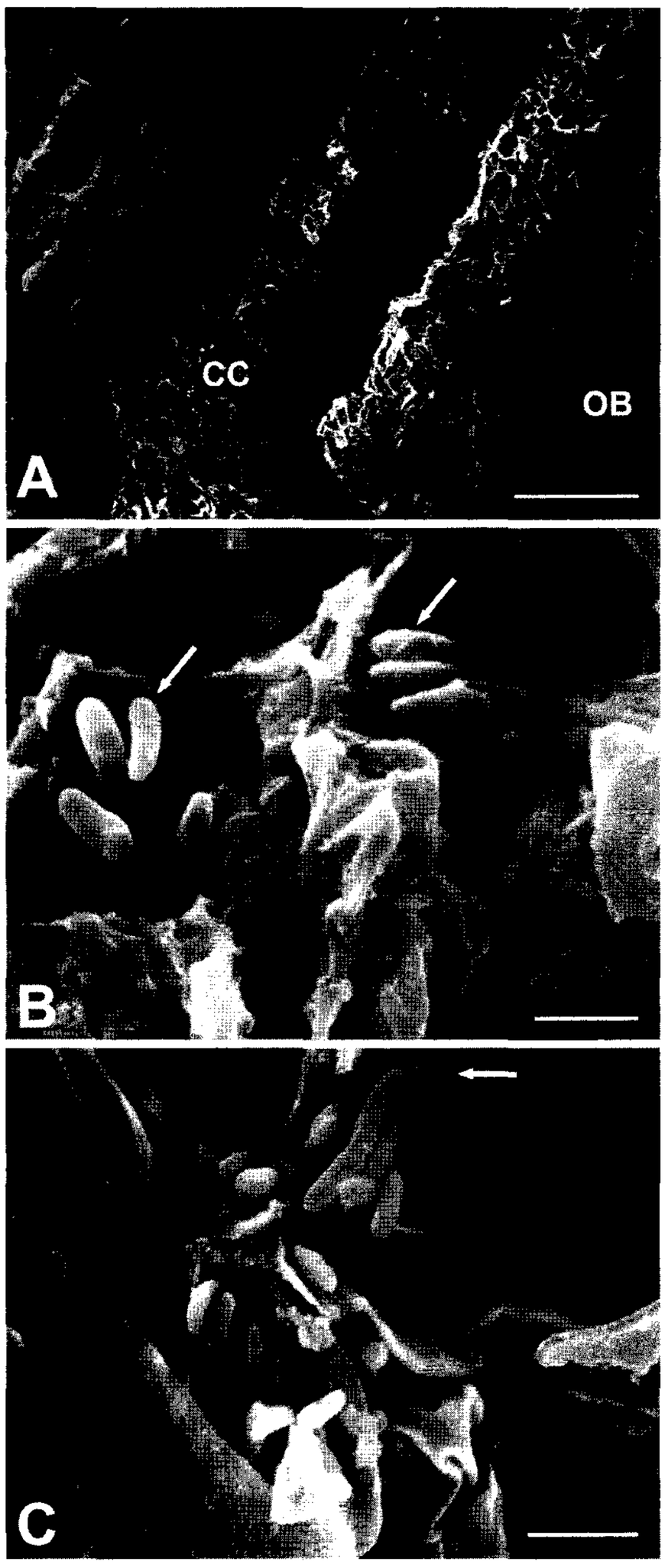

Fig. 4. Scanning electron micrographs of *F. circinatum* on stems of *P. densiflora*. (A) Uninoculated outer bark (OB) surface and cork cells (CC). Bar=500 μm. (B) Ungerminated microconidia (arrows) on the cork cell surface 2 hr after inoculation. Bar=5 μm. (C) Conidial germination on the cork cell surface 8 hr after inoculation. An arrow indicates a germ tube. Bar=10 μm.

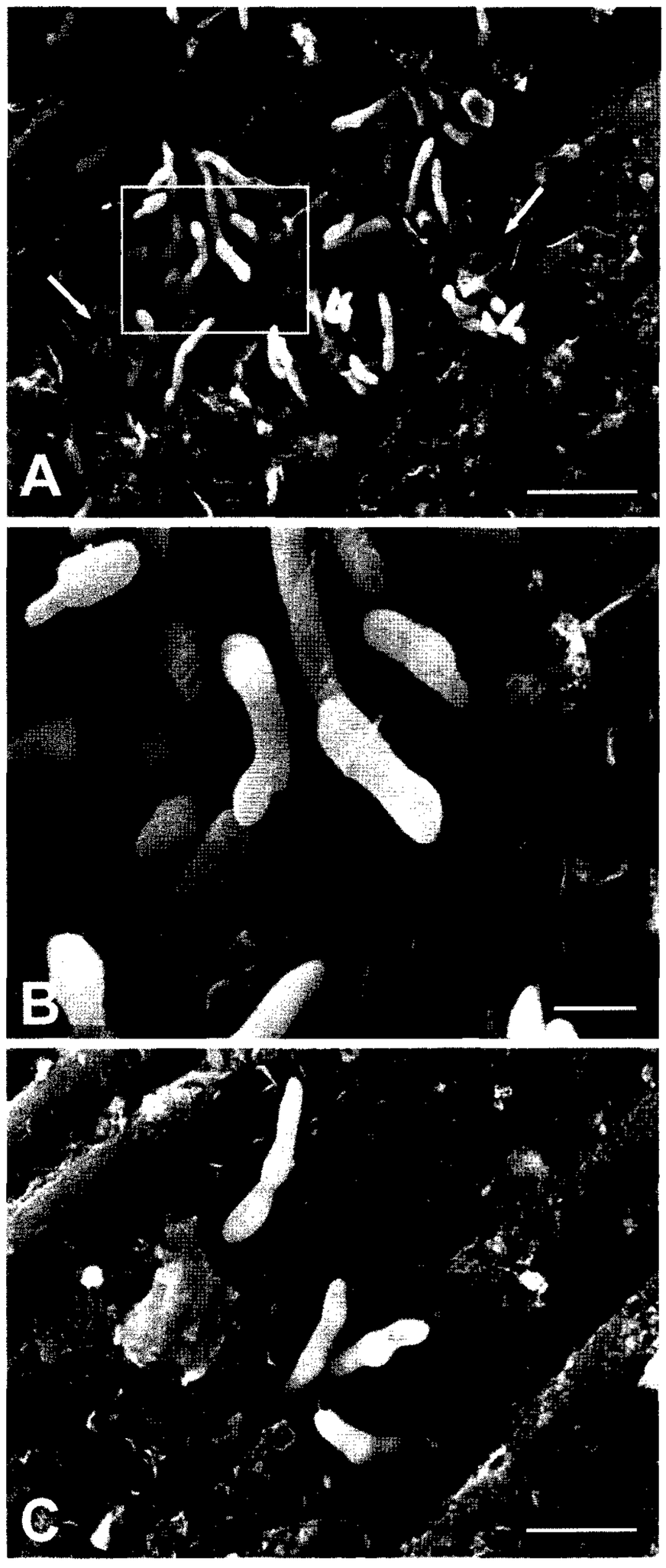

Fig. 5. Scanning electron micrographs of *F. circinatum* on stems of *P. densiflora*. (A) Conidial germination on the bark surface 12 hr after inoculation. Arrows indicate the degraded cuticle. Bar= 20 μm. (B) Higher magnification of the rectangle of 5A. Bar=5 μm. (C) Conidial germination on the bark surface 12 hr after inoculation. Bar=10 μm.

Fungal behavior on stems of *P. densiflora*. Similar results were found in *P. densiflora* as shown in *P. rigida*. Uninoculated outer bark surface of *P. densiflora* showed the cork cells exposed to the outside (Fig. 4A). Microconidia of *F. circinatum* were deposited on stems and measured approximately 5 μm long (Fig. 4B). A few conidia germinated 8 hr after inoculation (Fig. 4C). The outer bark surface was partially covered with the cuticle including epicuticular wax (Fig. 5A). Cuticular degradations were frequently observed on the bark surface, showing several holes and cracks. Some germ tubes appeared to grow through the small holes of degraded cuticle (Fig. 5B). Some microconidia were deposited between ridges on the outer bark surface (Fig. 5C). The cuticle appeared to be eroded either

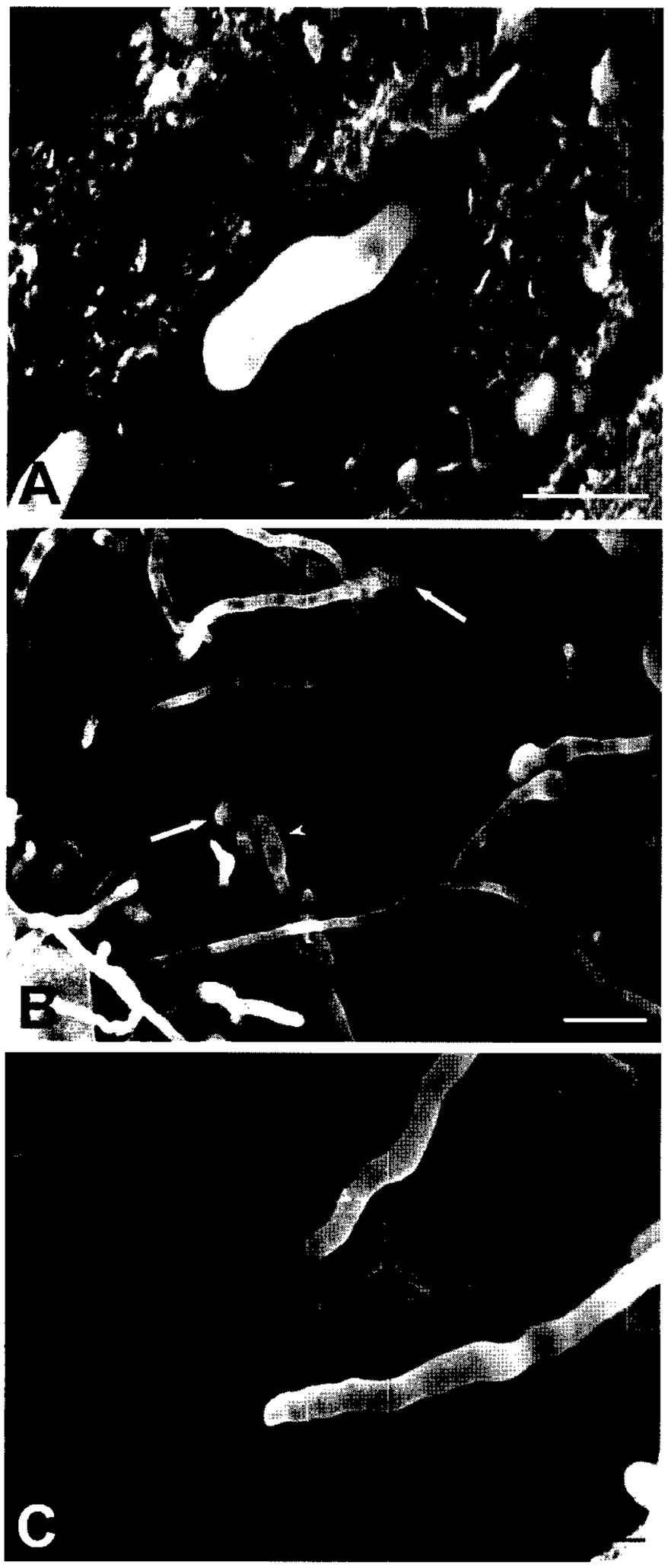

Fig. 6. Scanning electron micrographs of *F. circinatum* on stems of *P. densiflora*. (A) Conidial germination on the bark surface 12 hr after inoculation. Bar=5 μm. (B) Conidial germination on the bark surface 16 hr after inoculation. Arrows indicate appressoria-like structures. An arrowhead denotes a conidium. Bar=10 μm. (C) Conidial germination on the bark surface 16 hr after inoculation. Bar=5 μm.

at the tip of germ tubes or around germlings (Fig. 6A). Germ tubes grew over the stem surface and often formed appressoria-like structures, exhibiting rather swollen tips of germ tubes (Fig. 6B). Moreover, host cell wall cracks were found at the tip of germ tubes on the outer bark surface (Fig. 6C). Other germ tubes directed their growth to indented areas between ridges on the outer bark surface.

Discussion

This study increased our knowledge of the infection process of *F. circinatum* on pine stems in pitch canker development. The cellular differentiation, growth, and host entrance of *F. circinatum* on pine stems were described in this study. Furthermore, the use of scanning electron microscopy enabled us to document the fungal behavior with three-dimensional topography at a higher resolution than that of conventional light microscopy. There seems to be no significant differences in the pre-infection behavior of *F. circinatum* on stems between the two pine species. The sequential events of cellular differentiation such as conidial germination, germ tube elongation, and host entrance through cracks were overall similar to each other.

F. circinatum has been considered to be a wound pathogen. Inoculation areas are usually wounded for providing infection courts in pathogenicity tests (Enebak and Stanosz, 2003; Lee et al., 2000). In this study, possible infection courts of *F. circinatum* for entry into host tissues were evident on pine stems. In particular, wide spacing of cork exposed on the outer bark surface implies that the pathogen enter pine stems easily under favorable conditions in the field where severity of wounding and inoculum potential are already present. Surface cracks were also apparently entrance sites for *F. circinatum* on stems of the two pine species. Although it has not been yet described on their ontogeny in this study, surface cracks were often encountered with germ tubes, and appeared to have sufficient dimension for hyphal ingress. Infection of unwounded stems by the pathogen could be attributed to hyphal entrance into host tissues through surface cracks. It has been hypothesized that *F. circinatum* may remain dormant within the host without causing symptoms for an extended period of time (Schweigkofler et al., 2004). Such an endophytic stage would explain the observation that uninfested seeds from diseased branches gave rise to infected seedlings in Monterey pine (*P. radiata*) (Storer et al., 1998). Elucidation of the fate and mobility of *F. circinatum* hyphae within asymptomatic and unwounded host tissues awaits further research.

Host cell wall cracks and cuticular alterations were often found at the tip of germ tubes on pine stems in this study. These observations suggest that cell wall/cuticle degrading enzymes might have secreted from the germ tubes, dissolving the surrounding host regions. Endopolygalacturonases produced by *F. circinatum* have been suggested to be involved in pitch canker development (Chimwamurombe et al., 2001). Concomitantly, chemotactic interactions between host and pathogen may be plausible to support the growth of germ tubes to surface cracks on pine stems, as proposed in the cases of wounded areas of apple stems (Brown and

Hendrix, 1981) and surface cracks on apple fruits (Kim et al., 1999). However, this study does not exclude the possibility that either cracks or cuticular alterations predate the germ tube elongation, and the germ tubes may be placed on cracked or altered areas. It is likely that the cuticle degradations accompanied with holes and cracks could be attributed to the chemical treatment and heating process during critical point drying of plant tissues prior to scanning electron microscopy (Jorgensen et al., 1995).

An intriguing finding in this study was the formation of appressoria-like structures on pine stems. The morphological indications of appressoria-like structures were cessation of germ tube growth and swelling of germ tube tip. The appressoria-like structures were strikingly similar in shape to those observed in GFP-labeled hyphae of *F. graminearum* in barley and wheat spikes (Jansen et al., 2005) and *F. oxysporum* on melon roots (Inoue et al., 2002). To our knowledge, this is the first report on the formation of appressoria-like structures by *F. circinatum*. The occasional occurrence of appressoria-like structures of *F. circinatum* on pine stems may imply that appressoria-like structures are not essentially required for pathogen infection. Future work remains to be done to characterize the triggering factors for the formation of appressoria-like structures of *F. circinatum*, possibly involved in fungal pathogenicity and survival. The knowledge of the development and regulation of fungal structures will provide a more representative picture of the structure-function relationships of plant pathogenic fungi with host plants.

Acknowledgments

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