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Full Length Research Paper

Infection and establishment of *Ascochyta anemones* in leaves of windflower

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Leaf spot, caused by *Ascochyta anemones*, is one of the major diseases of windflower in China. Histological studies of the artificially inoculated leaf tissues with *A. anemones* conidia were observed by light and electron microscopy to elucidate the host-pathogen interaction. Conidial germination and germ tube formation began after 4 h of inoculation. Each conidium produced one germ tube which penetrated the host surface. The fungus gain entry of the host by direct penetration of the leaf cuticle, following the formation of appressorium after 12-24 h of inoculation. Most appressoria were formed in the grooves between adjacent epidermal cells. Once the fungus was fully established, it destroyed internal tissues, resulting in diseased lesions on the leaves after 3-6 days of inoculation. Development of leaf spots and fungal pycnidia could be observed on necrotic areas within 7-14 days after inoculation.

Key words: Windflower, *Ascochyta anemones*, infection process.

INTRODUCTION

Windflower (*Pulsatilla* sp.) is a perennial medicinal plant in the *Ranunculaceae* with high economic as well as medicinal value in China. It is a commonly used traditional Chinese medicine (Pan et al., 2004; Bang et al., 2005). The leaf spot of windflower, caused by *Ascochyta anemones*, has been recorded in China (Yu et al*.,* 2008). Symptoms occur on infected leaves and stems, round spots with brown margin, where pycnidia are arranged in concentric rings. As the disease progressed, infected plant parts begin to wilt and then die. The morphological and cultural characteristics of *A. anemones* has been described (Fu et al., 2010), but the histological and ultrastructural aspects of the infection by *A. anemones* have not been documented.

Entry of fungal pathogens into plants may proceed through direct penetration or mechanical pressure or indirectly through wounds or natural openings such as stomata. Different species may display different modes of entry under different conditions. Infection process by *A. anemones* was studied in detached windflower leaves by artificial inoculation. The objective of the research was to understand conidial infection on the host tissue and to elucidate the host-parasite interaction.

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MATERIALS AND METHODS

Plant materials

A highly susceptible windflower (*Pulsatilla koreana* Nakai), kindly provided by horticulture department, Shenyang Agricultural University, Liaoning, China, was used in the present experiments. Plants were grown in a temperature controlled greenhouse (18-25°C) under natural daylight. The plant materials were used for artificial inoculation at 6-10 growth leave stage.

Pathogen

Cultures of *A. anemones* were isolated from naturally infected leaves and incubated on potato dextrose agar PDA medium (200 g potato, 20 g dextrose, 20 g agar, 1 L distilled water) at room temperature. Based on pathogenicity test, morphological characteristics and the sequence of ribosomal DNA-ITS, the pathogens were identified as *Ascochyta anemones.* After 2 weeks of incubation, a conidial suspension was made by addition of sterile

distilled water onto the Petri plate. Mycelium was removed by filtration through 3 layers gauze. Conidial suspension was adjusted to 1×10^5 conidia ml^{-1} with the aid of a hemacytometer.

Conidial germination on glass slides

The conidial suspension 20 µl was pipetted onto glass slides. They were incubated at 25°C inside Petri dishes containing distilled water to maintain 100% relative humidity (RH), and the plates were sealed with parafilm to prevent dryness. The slides were examined with a light microscope at 4 h intervals up to 40 h for germination. At least 100 conidia were examined at each interval. This experiment was carried out in triplicate.

Artificial inoculation

Adolescent plants of windflower were inoculated by using atomizer in the evening with above mentioned suspension and then covered with a plastic bag to uphold a high humidity conditions for at least 24 h. The inoculated plants were placed at 25°C in the greenhouse under natural photoperiod.

Light microscopy

The inoculated leaf blade was removed carefully and cut into pieces of approximately 5 mm^2. The samples were stained with trypan blue-lactophenol for 10 min, and then decolorized in saturated trichloroacetal dehyde monohydrate, after 12 h. The trichloroacetal dehyde monohydrate was removed with a pipette and replaced with fresh sterile water. The cleared leaf squares placed on a glass slide and examined under a light microscope (Leica BME).

Scanning electron microscopy

The samples were fixed in 2.5% glutaraldehyde dissolved in 0.5 M phosphate buffer at pH 7.2 and stored overnight at 4°C, then rinse with the same buffer. After dehydration using a graded ethanol series, samples were critical-point dried in carbon dioxide after a graded transition from ethanol to acetone. Sections (5×5 mm) were mounted on stubs, coated with gold-palladium, and examined with a JEOL-JSM-T300 SEM operating at 15 kV.

Transmission electron microscopy

The infected tissue were cut into small pieces (1 × 2 mm) and fixed with 3% glutaraldehyde overnight. After two washings, samples were post-fixed with 1% osmium tetroxide for 2 h before being dehydrated in a graded ethanol series. Preparations were embedded in Spurr's epoxy resin. Thin sections (100 nm thick) were cut with glass knives and collected on gold grids. Sections were stained for 10 min with uranyl acetate and for 5 min with lead citrate for examination at room temperature. Sections were viewed with a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) at 60 kV.

RESULTS

Conidial germination on glass slides

The conidia started germinate on glass slides 4-6 h after inoculation, whose length did not exceed 30 µm. The conidia became turgid and slightly swelled with imbibition of water before germination. Germ tubes emerged from one pole or simultaneously from both cells. Sometimes the conidia became separated at the septum and each cell producing a germ tube. The germ tubes developed into branched, septate hyphae (Figure 1).

Light microscopy

Light microscopy (LM) observations of the fungus on detached leaves are presented in Figure 2. Within 24 h in the presence of free water, the spores developed germ tubes which grew on the leaf surface (Figure 2A, B). Some conidia have more than one germ tube originating from the same spore (Figure 2C). The direction of emergence of germ tubes appeared to be tropism in reaction to cell junctions and not directed towards stomata. A circular-shaped appressorium was the most common form of infection structure, usually appearing at the end of a germ tube. Most appressorium formed at the junctions between adjacent epidermal cells and penetrated the cuticle. The penetration of the stomatal pore by germ tubes was never observed, even when spores landed near the guard cells. Germ tubes regularly grew over or past stomatal pores without changing shape or never grew directly towards stomata (Figure 2D). Occasionally, penetration of the host by the fungus occurred without any light microscopically visible formation of an appressorium. The first symptoms on leaves 3 day after inoculation were round spots with brown margin, mature pycnidia were observed within these brown lesions 7-14 days after inoculation (Figure 2E).

Scanning electron microscopy

The scanning electron microscopy observation showed that conidia cylindrical with rounded ends, bicellular, medianly uniseptate, slightly constricted at the septum (Figure 3A). Spores began to germinate and germ tubes grew for varying distances along the leaf surface 4-24 h after inoculation (Figure 3B). At 2- 4 days after inoculation, pathogen produced many thriving branchy mycelia that cover the host surface. The penetration hyphae repeatedly branched multiseptate and ramified through the tissue (Figure 3C). After the degeneration of host tissue, pycnidia could be observed 7-14 days after inoculation of the leaf. Mature pycnidia were spherical in shape with thick dark or light brown coverings, measuring90-185 µm in diameter (Figure 3D).

Transmission electron microscopy

Initially, only a few epidermal cells were colonized by the fungus within the first day following inoculation. The

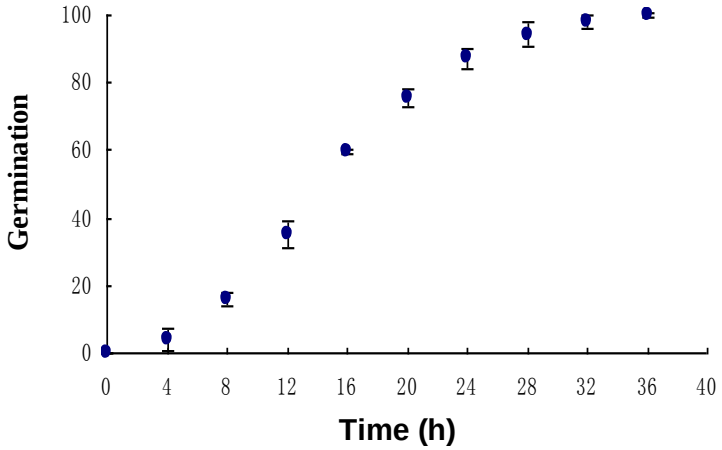

Figure 1. Time course for conidial germination of *A. anemones* of a conidial suspension on glass slides.

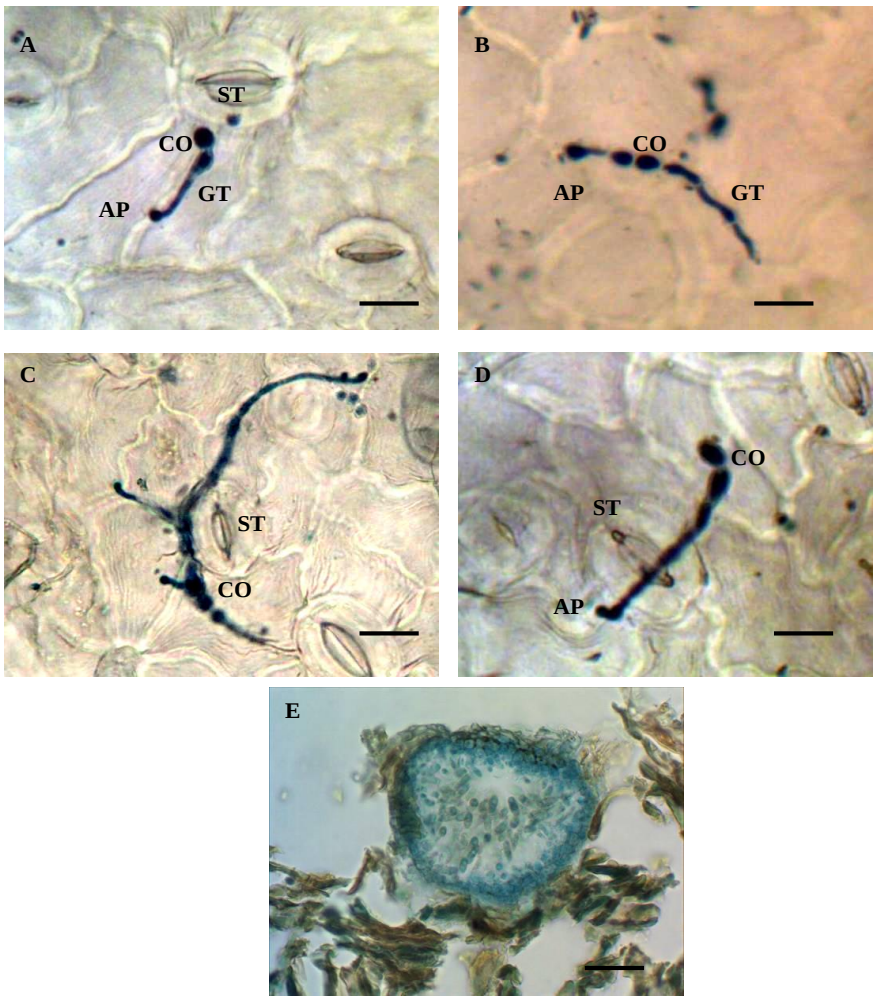

Figure 2. Light micrograph of *A. anemones* on windflower leaves. (A) Conidium with one germ tubes (scale bar=15 µm); (B) Conidium with two germ tubes (scale bar =15 µm); (C) Conidium with multi-tubes (scale bar =15 µm); (D) No penetration via stomata was observed (scale bar =15 µm); (E) Mature pycnidium with pycnidiospores (scale bar =50 µm) AP= appressorium, GT=germ tube, CO=conidium, ST= stomata.

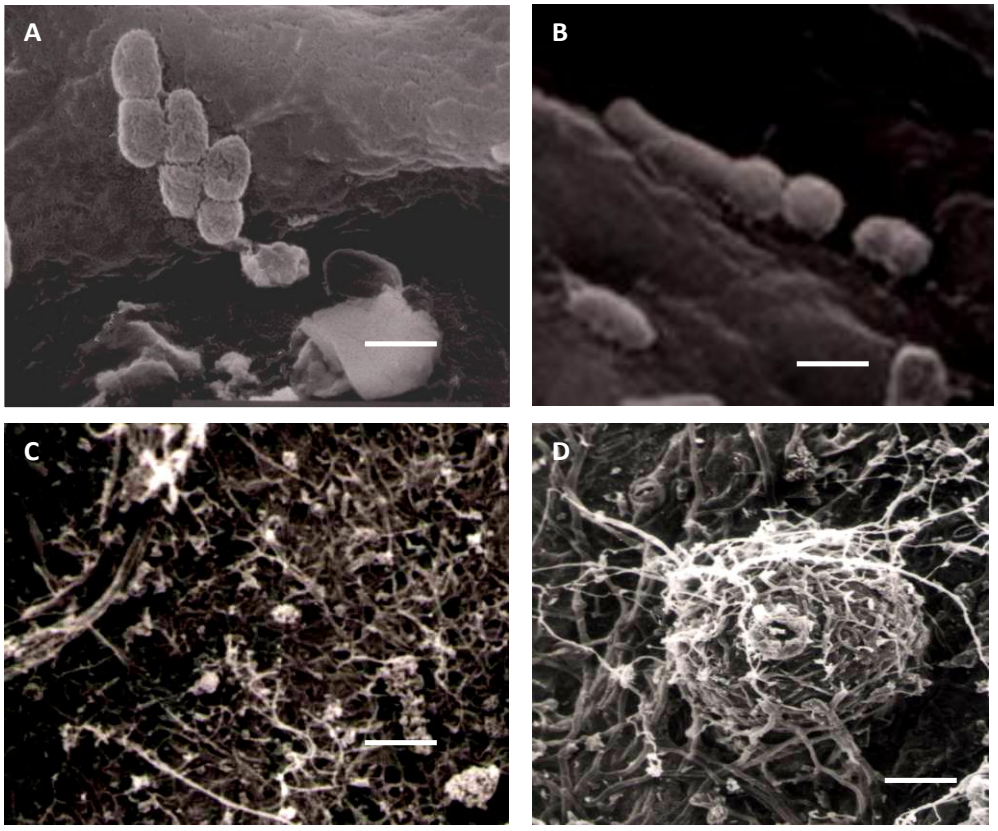

Figure 3. Scanning electron micrographs of *A. anemones* on windflower leaves. (A) Morphology of conidia (scale bar = 5 m). (B) Magnification of a germinated pycniospore (scale bar = 5 m). (C) Development of hyphae (H) at the surface of the cuticle (scale bar =80 µm). (D) Morphology of pycnidium (scale bar = 50 µm).

mesophyll cell usually showed a normal ultrastructure with chloroplast containing grana and stroma thylakoids (Figure 4A). With the development of intercellular hyphae at 3 days post-inoculation, the number of electron dense granules began to increase in the spaces between the wall and the cytoplasm membrane and in the central cell. At the same time, the envelope membrane of the chloroplasts broke and the chloroplasts deformed (Figure 4B). With the development of intercellular hyphae at 5 day after inoculation, many vesicles appeared and gradually fused into a bigger vacuole. Pycnidia originated from an aggregate of fungal hyphae amongst the disintegrated host tissue (Figure 4C). The detached leaves were severely damaged at later stage. *A. anemones* favored colonization of the tissues by formation of pycnidia once the tissue was infected. In pycnidia, spores appeared different morphologically, and degraded organelles of host cells mingled often at 7-14 days after inoculation (Figure 4D).

DISCUSSION

The infection and establishment of *A. anemones* in leaves of windflower was studied by light microscopy, SEM and TEM. The behavior of *A. anemones* on the surface of windflower showed several features, such as tropism of germ tubes in reaction to cell junctions and aspersoria formation, which are also found in many other fungal species (Graaf et al., 2002; Nicholson et al., 1991).

Pathogen fungi can produce a wide array of cellular structures specialized for cell wall penetration, such as appressoria and haustoria (Mendgen and Deising, 1993). In our study, direct penetration of *A. anemones* to leaf tissues through the cuticle was observed with the formation of appressoria. In the previous studies, penetration with appressorium were also reported for other *Ascochyta* species, such as *Ascochyta rabiei* (Höhl et al., 1990; Pandey et al., 1987), *Ascochyta fabae* (Maurin et al., 1993), *Ascochyta pisi* (Heath et al., 1969) and also in *Phoma exigua* and *Phomopsis helianthi* (Muntanola-Cvetkovic et al., 1989). Maurin et al. (1993) studied the histopathology of interaction between *Ascochyta fabae* and *Vicia faba*. His observations were in accordance with those for *A. anemones*. In both species, the spores developed more or less branching germ tubes which grew on the leaf surface. The germ tubes tips were swelled and developed to form an appressorium which

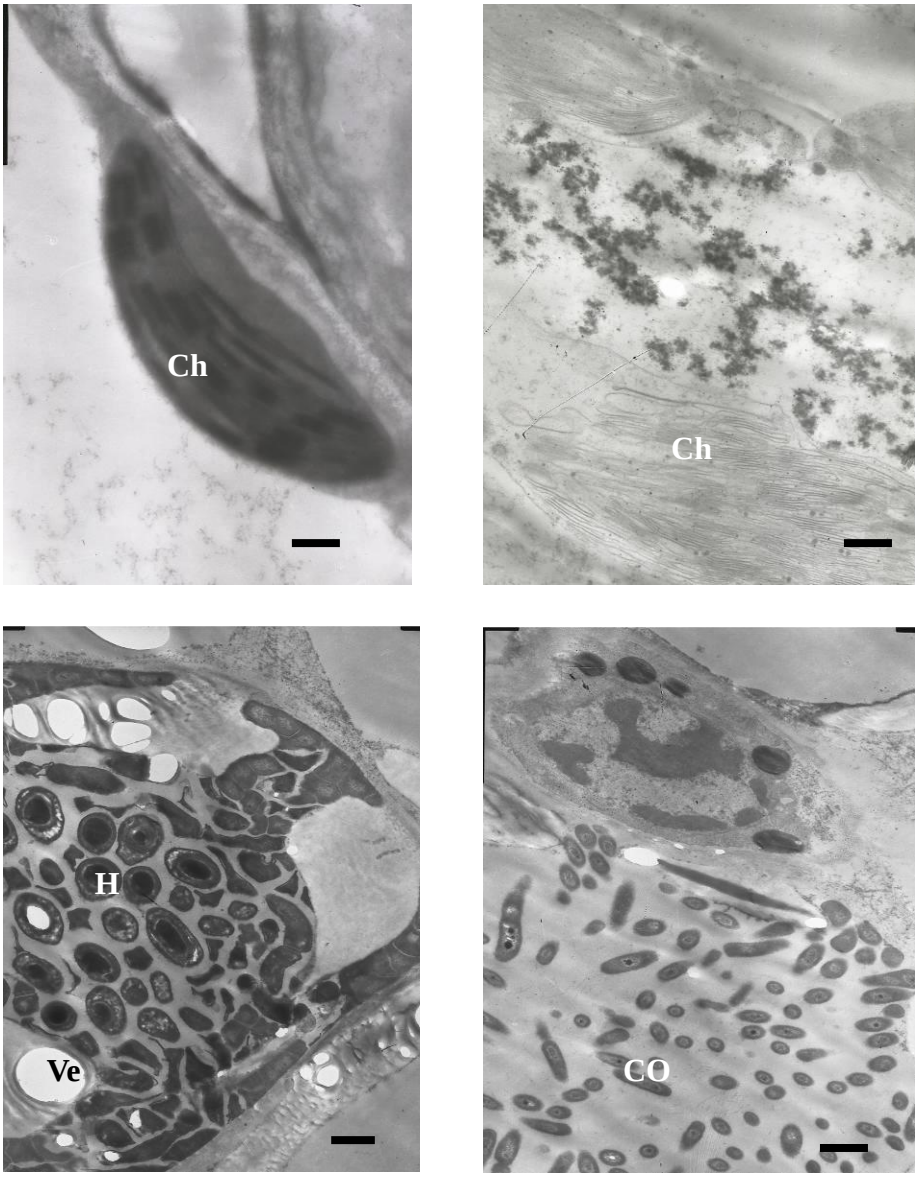

Figure 4. Transmission electron micrographs of *A. anemones* on windflower leaves. (A) Normal ultrastructure with chloroplast at 1 day after inoculation (scale bar = 500 nm); (B) Electron dense granules increase and the envelope membrane of the chloroplasts broke at 3 day after inoculation (scale bar = 500 nm); (C) Disintegrated mesophyll tissue mainly occupied by hyphae of *A. anemones* at 5 day after inoculation (scale bar = 1 µm); (D) Pycnidial spores were morphologically different on necrotic areas (scale bar = 1 µm). Ch= chloroplast, CO=conidium, Ve=vesicle, H= hypha.

penetrate the host cuticle directly. In contrast, penetration without appressorium was reported in other fungi closely related to *Ascochyta*, such as *Phoma linguam* (Hammond et al., 1985), *Phoma narcissi* on Hippeastrum leaves (Saniewska et al., 1997), *Phomopsis longicola* (Baker et al., 1987), *Phomopsis leptostromiformis* on the stem of *Lupinus angustifolius* (Williamson et al., 1991), and *Septoria apiicola* (Donovan et al., 1990). Some factors like epicuticular waxes, rigidity, and surface hardness affects the formation of appressoria (Höhl et al., 1990).The ultrastructural results showed that fungal hyphae were aggregated to form pycnidia in leaflets after 7 -14 days of inoculation, which corroborates with observation made by Llarslan et al. (2002).

Ascochyta blight fungi like *A. rabiei* produces phytotoxins and enzyme. This fungus *A. rabiei,* produces three types of solanopyrones; A, B and C (Hohl et al., 1991). Solanopyrones A, B, C were first found to be products of *Alternaria solani*, the causal agent of late blight of potato (Jayakumar et al., 2006). In moderately or

severely blighted leaves, the production of chlorophyll ‘a’ and chlorophyll ‘b’ was reduced significantly (Gaur, 2000). This was attributed to inhibition of production by *A. rabiei* or enhanced activity of chlorophyllase. The role of phytotoxins and enzyme produced by *A. anemones* as possible factor of virulence during infection and establishment by the fungus which is still to be determined.

Further histopathological studies of the mode of penetration and tissue colonization at different parts of windflower, such as the base of the stem, leaf petiole or leaf lamina, are necessary to elucidate the *Ascochyta*-windflower interactions, using both tolerant and susceptible genotypes.

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