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ORIGINAL PAPER

Natural infectious behavior of the urediniospores of *Melampsora larici-populina* on poplar leaves

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Abstract The uredinial stage in the life cycle of *Melampsora larici-populina* on poplar leaves is the most important pathogenic phase. We captured partial phases of uredinial infection in the wild, aiming to reconstruct the process of uredinial ontogeny by using scanning and transmission electron microscope. At the initial infection stage, germ tubes germinated from the echinulate urediniospores. Germ tubes were frequently seen to merge with the leaf surface and cuticle breakage was observed, indicating direct hyphal penetration. Stomata penetration occurred commonly, sometimes with more than one germ tube penetrating the same stoma. *Melampsora larici-populina* did not form appressoria in the infection process, implying that infectious behavior of this pathogen may differ from the other rust pathogens. In general, germ tubes branched randomly, and no distinct evidence indicated that stoma could induce or orient germ tube branches. However, oriented germ tube growth has been occasionally observed in other studies. The urediniospores collapsed and finally wizened when they became nutrient stressed. At the last stage of infection, the uredinia erupted from the leaf epidermis and appeared as orange pustules on the leaf surface.

Keywords *Populus deltoides* · *Melampsora larici-populina* · Germ tube · Infectious behavior

The online version is available at http://www.springerlink.com

Corresponding editor: Hu Yanbo

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Introduction

Populus deltoides naturally occurs in Canada, from eastern Alberta to Quebec, the southern United States, including Florida, Texas, and Arizona, and northern Mexico (Kartesz and Meacham 1999). *Populus deltoides* is a valuable timber species widely used in short-rotation intensive culture (SRIC) in the United States and Canada (Bull and Muntz 1943). This species has also been introduced as an SRIC worldwide. *Populus deltoides* was introduced to China in the early 1970s. Presently, this species and its hybrids dominate forestry plantations in areas along the middle and lower reaches of the Yangtze River. However, an outbreak of rust disease has been observed in poplar plantations since the 1990s and is gradually becoming a major problem hampering the development of poplar plantations in southern China (Wan et al. 2013). Based on morphological characteristics and analyses with species-specific molecular markers, the rust pathogens causing these outbreaks were identified as *Melampsora larici-populina* (Wan et al. 2013).

Melampsora larici-populina is the most devastating, widespread pathogen infecting commercially important poplar species (Steenackers et al. 1996; Tian et al. 1999). It features frequent pathogenic variations and an impressive adaptive potential (McDonald and Linde 2002; Tian et al. 2009). Poplar leaf rust disease caused by this specific race can lead to more than a 50 % reduction in annual growth (Covarelli et al. 2013). Repeated infections over successive years may result in the complete loss of a poplar plantation (Cervera et al. 1996). To limit the damage resulting from this pathogen, great efforts have been made by scientists to develop new breeding strategies. Besides generating a collection of hybrid poplars with complete resistances to *M. larici-populina* (Newcombe et al. 1996; Villar et al. 1996; Lefévre et al. 1998), poplar breeders have attempted to uncover the host-pathogen interactions (Shain and Jarlfors 1987; Laurans and Pilate 1999; Tian et al. 2001) and to identify molecular markers for resistance against *M. larici-populina* (Cervera et al. 1996; Jorge et al. 2005). Recently, a combined genomic and transcriptomic approach led to the discovery of a very large repertoire of candidate *M. larici-populina* effectors (Duplessis et al. 2009, 2011; Hacquard et al. 2011). Despite considerable efforts by breeders to generate resistant hybrid poplars, rapid breakdown of resistance was generally observed because of the continuous development of new rust pathogens (Shang et al. 1986; Newcombe et al. 2000; Liang et al. 2006). Thus, the sustainability of newly selected resistance-types requires better understanding of the interactions between *Populus* and *M. larici-populina*.

In the life cycle of *M. larici-populina*, the uredial stage on poplar leaves constitutes the most important phase for the pathogen (Mlodzianowski et al. 1978). Under favorable conditions, urediniospores form on poplar leaves and rapidly infect neighboring trees. During the infection process, the urediniospores germinate 24 h after inoculation of the poplar (*P. deltoides*) leaf. After 36 h, haustorium forms, which involves the redirection of the host's metabolism and the suppression of host defenses (Tian et al. 2001). This infection structure is essential for the establishment of a successful bio trophic relationship (Heath 1997; Voegele et al. 2009). Thereafter, a slow expansion of colony size is observed approximately 72 h after inoculation (Tian et al. 2001). At approximately 168 h, uredinia erupted from the leaf epidermis and appeared as orange pustules on the leaf surface, leading to another cycle of infection (Hacquard et al. 2010). The development of rust disease reduces the assimilation surface of the infected trees (Mlodzianowski et al. 1978).

Melampsora larici-populina is a rust pathogen that has recently spread to areas in southern China (Wan et al. 2013). In this study, we aimed to record and reconstruct the process of pathogenesis and the uredinial ontogeny of *M. larici-populina* on the susceptible host by using scanning and transmission electron microscopy (SEM and TEM, respectively). This work provides information to better understand the *Populus–M. larici-populina* interaction and verifies the findings of previous studies.

Materials and methods

In mid-October, 2011, rust- infected mature leaves of a susceptible *P. deltoides*, clone "S3503", were collected. This clone was imported from Stoneville, Mississippi, USA, in February, 1991, and was maintained in the poplar nursery on the campus of Nanjing Forestry University. In the field, this *P. deltoides* clone was consistently observed to be highly susceptible to *M. larici-populina* (Wan et al. 2013).

For the SEM examination, urediniospores were air-dried, mounted on aluminum stubs and sputter-coated with approximately 30 nm of gold/palladium. The specimens were examined with a JEOL 840A SEM (JEOL, Tokyo, Japan). For the TEM examination, small leaf segments (1 mm × 2 mm) were fixed in 3 % glutaraldehyde and 2 % formaldehyde in 0.1 M phosphate buffer (pH 7.2), vacuum infiltrated and stored in the primary fixative at 4 °C for 24 h after sample collection. Specimens were transferred to fresh buffer (three washes for 30 min) and post-fixed for 30 min to 1 h in 1 % osmium tetroxide (OsO_4) at 4 °C. Following three further buffer washes, the fixed material was dehydrated in an acetone series (25, 50, 75, 95, 2 × 100 %), infiltrated and embedded in Polarbed 812 epoxy resin (cured 72 h at 6 °C). The grid-mounted sections were stained with saturated uranyl acetate in 50 % ethanol (3 min), washed in 50 % ethanol and distilled water, then stained with lead citrate (3 min) in distilled water. The processed specimens were examined with a Hitachi-600A (Hitachi, Ltd., Tokyo, Japan) microscope.

Results and discussion

Germ tube development

At the initial stage of symptom development, *M. larici-populina* uredinia emerged as yellow-orange pustules of 1–2 mm diameter on the abaxial leaf surface. Then, the uredinia produced a large number of asexual urediniospores. Under the SEM, the wall surface of the urediniospore appeared echinulate except for a smooth patch at the broad apex (Fig. 1). Germ tubes germinated from the echinulate urediniospores. The walls of the hydrated *M. larici-populina* urediniospores were partially deliquescent, facilitating germination of the germ tubes (Rijkenberg 1972). Germ tubes of *M. larici-populina* were rope-like in shape and clingy

Fig. 1 Detailed morphological characteristics of *Melampsora larici-populina* urediniospore
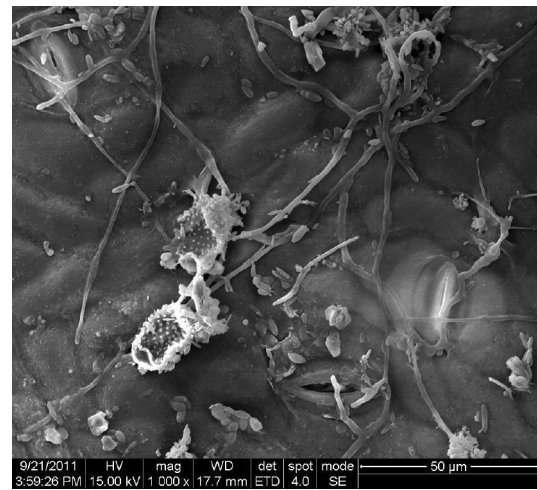

Fig. 3 Wizened and collapsed *Melampsora larici-populina* urediniospores

Fig. 2 Germ tube sprouting from a *Melampsora larici-populina* urediniospore
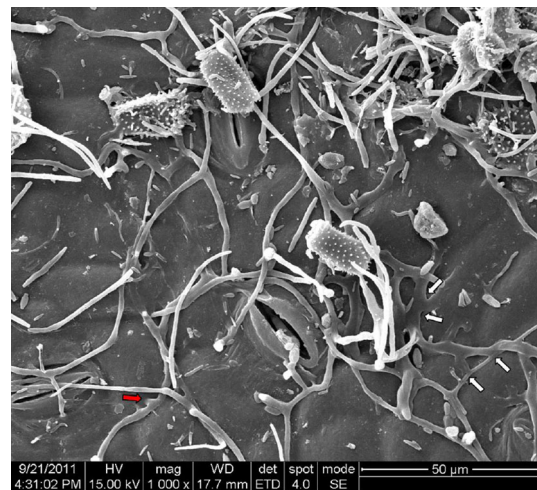

Fig. 4 The dendritic network formed by *Melampsora larici-populina* germ tubes on a poplar (*Populus deltoids*) leaf surface. White arrows indicate the germ tubes merging with each other, and the red arrow indicates the germ tube penetrating the cuticle directly

when appressed to the leaf surface (Fig. 2). Spiers and Hopcroft (1988) reported that individual urediniospores contained four germ pores, forming four germ tubes. We found that one urediniospore generally formed one to three germ tubes but only occasionally formed four germ tubes.

As germ tubes extended, they branched several times to form a dendritic network on the leaf surface (Figs. 3, 4). Then, after penetration of the germ tubes, haustoria formed, and the urediniospore became the haustorial mother cell (Spiers and Hopcroft 1988; Tian et al. 2002). The cytoplasm of the mother cell migrated continuously into the haustorium, causing the mother cell to become highly vacuolated and it ultimately collapsed (Spiers and Hopcroft 1988; Tian et al. 2002). In this study, we also observed many collapsed and wizened mother cells (Fig. 3).

Direct penetration

Germ tubes varied in morphology when merged with each other, fusing into swollen lizard- or octopus-shaped structures (Figs. 4, 5). Some germ tubes merged with the epidermis of the poplar leaf, and directly penetrated the cuticle (Figs. 4, 5). Cavities and broken cuticles were found at the infection sites (Fig. 5), similar to observations reported by Yu et al. (2011). Spiers and Hopcroft (1988) found a distinguishable hole was made by hyphae through direct penetration; however, no infection structure was formed under the hole. We observed that direct penetration occurred only occasionally. Together with the observation of Spiers and Hopcroft (ibid.), this indicated that direct
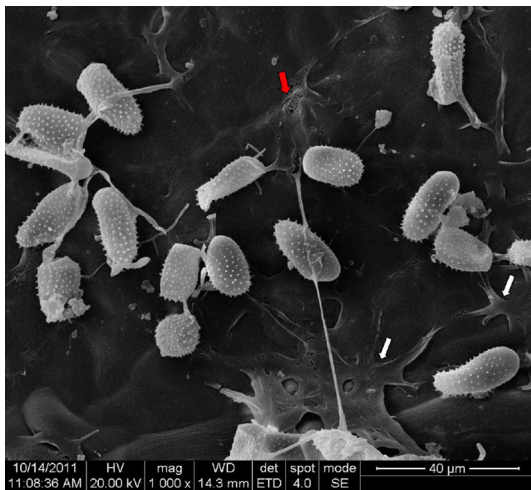
Fig. 5 Typical fused cells of germtubes from different *Melampsora larici-populina* spores. White arrows indicate the germtubes merging with each other and forming a swollen lizard- or octopus-shaped structures. The red arrow indicates the broken poplar (*Populus deltoids*) leaf cuticle
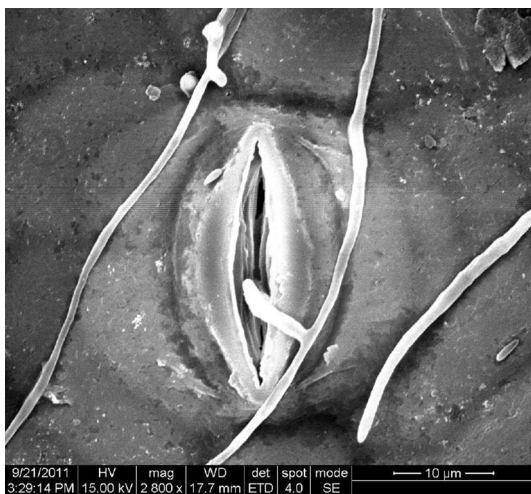

Fig. 6 The *Melampsora larici-populina* germ tubes passed by the stoma but short branch penetration occurred

penetration might not be the main infection path. However, the role of direct penetration remains unclear. Yu et al. (2011) suggested direct penetration of epidermal cells could cause leakage of cell contents, resulting in increased humidity around germ tubes that could reduce the frequency of occurrence of fused germ tubes, which typically formed under low ambient humidity. Additionally, increased humidity could also increase the survival of germ tubes in arid environments (Yu et al. 2011).

Stomatal penetration

Germ tubes extended on the leaf surface, with clear stomatal invasions, were frequently observed (Figs. 6, 7),

Fig. 7 Two *Melampsora larici-populina* germ tubes enter the same stoma

although germ tubes did not take the most direct route to the stomata. They were frequently observed passing over stomata without penetrating (Figs. 6, 7), but nearby short branches entered opened stomata. The penetration of a single stoma by two germ tubes was commonly observed (Fig. 7). Increased magnification revealed that the germ tubes branched randomly (Figs. 3, 4), and there was no observable indication that germ tubes branched more frequently when near stomata.

In the infection process, the host surface appears to play a crucial role in determining how germ tubes and infection structures develop (Maheshwari et al. 1967). Wemer (1982) considered that stomal recognition by *M. larici-populina* was dependent on the specific sculpturing of the surface of the stomatal apparatus and the structure of the guard cells. However, no specific orientation of the *M. larici-populina* germ tubes on the host was observed and they appeared to randomly penetrate stoma (Spiers and Hopcroft 1988), like those of some other rusts (Longo et al. 2000; Vaz Patto and Niks 2001; Vaz Patto et al. 2009). Whether the leaf surface affects germ tube orientation remains to be determined. Yu et al. (2011) observed that some long germ tubes of *M. larici-populina* occasionally rolled back near the stomata and formed an infection structure entering the stomata. Thus, more evidence is needed to clarify whether there is an unknown mechanisms triggering stomatal recognition by *M. larici-populina*.

Evidence from appressorium induction by artificial membranes suggested that at least eight rust species formed appressoria in response to thigmotropic stimuli (Heath 1997). However, germ tubes were observed to penetrate stomata without forming clearly defined appressoria (Figs. 6, 7). Chiba (1964), Taris (1968), and Wemer (1982) observed that *M. larici-populina* did not form appressoria. In a study including *Melampsora medusae* and *M. larici-*

populina, Spiers and Hopcroft (1988) reported that *M. medusae* commonly formed appressoria over stomata; however, *M. larici-populina* did not form appressoria. Since most available studies indicated no appressoria formation during the infection process of *M. larici-populina*, this species' infectious behavior may differ from other rust pathogens.

Most of the early studies describing the penetration and establishment of *Melampsora* rust infection in poplars reported that germ tubes penetrated leaves exclusively via stomata (Chiba 1964; Taris 1968; Mlodzianowski et al. 1978; Wemer 1982). In contrast, Omar and Heather (1976) concluded that the germ tubes of *M. larici-populina* penetrated leaves directly and very few entered via stomata. Spiers and Hopcroft (1988) observed direct penetration, but this was rare and most germ tubes penetrated leaves via stomata. Yu et al. (2011) observed more direct penetrations compared with those via stomata. Thus, previous reports on the manner of penetration were different, leading to controversy. In this study, we observed both direct and stomatal penetration, with the latter occurring more commonly. The urediniospore germ tube must form the infection structures for infection to occur (Rossetti and Morel 1958; Maheshwari et al. 1967). Obvious infection structures were observed after the hyphae penetrated a stoma (Spiers and Hopcroft 1988), thus the role of stomatal penetration was confirmed. As for direct penetration, it did not result in infection structures (Spiers and Hopcroft 1988), and its role in the infection process remained unclear.

After the hyphae penetrated the leaf tissue, the palisade cells in the leaves of *P. deltoides* became shrunken and vacuolated (Fig. 8). Finally, the uredinia erupted from the leaf epidermis and were seen as orange pustules. In the uredinia, the wall surface of the newly formed urediniospore was smooth at the initial stage, and became echinulate with the development of urediniospore (Fig. 9). Urediniospores were eventually released and disseminated to start another infection process. A complete cycle of infection took about 168 h (Hacquard et al. 2010). In a short period of time, the polycylic production of urediniospores occurred, leading to severe epidemics on poplar leaves (Barrès et al. 2008). Uredinia were uniformly distributed on the foliar surface covering the entire leaf blade but did not develop on leaf veins.

Chiba (1964) and Mlodzianowski et al. (1978) examined rust infections (*M. larici-populina*) on poplars, demonstrating that the host cultivar significantly affected all facets of rust infection, including the rate and extent of urediniospore germination, the extent and pattern of germ tube extension, and whether stomata were penetrated. However, these studies were only performed on a highly susceptible host. Clearly, it is essential to investigate the
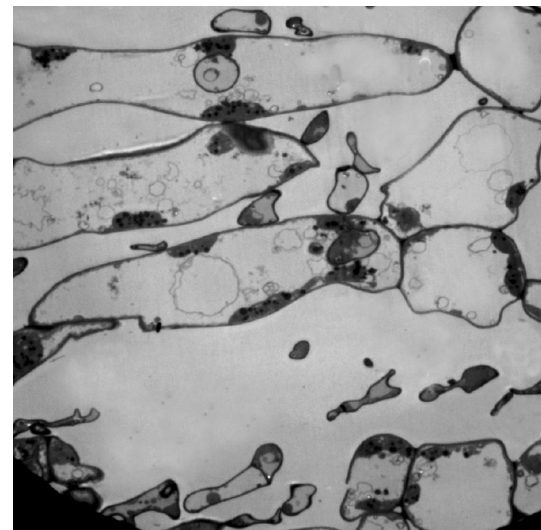
File name =895•gb•018.bmp;Image date=2011/10/31 10:33:57;Image number=4237;Image comment=Hitachi TEM system; Calibration=1.750nm/pixel at ×10.0k;Magnification=×0.3k;Lens mode=Zoom•1;Spot number=5;Image rotation=0°; Acc.voltage =80kV;Emission=9.8µA;Stage X=277Y=737Tilt angle=0.0;Azim angle=0.0

Fig. 8 The hyphae of *Melampsora larici-populina* ramifying intercellularly throughout the palisade tissue of *Populus deltoids*
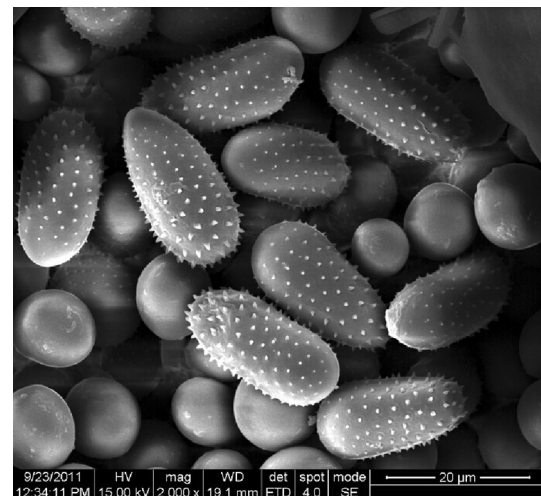

Fig. 9 Newly formed *Melampsora larici-populina* urediniospores with smooth wall surfaces and an echinulate urediniospore with a smooth patch at the broad apex

development of the infection structures of *M. larici-populina* on poplar cultivars varying in rust resistance. Modern genetics detected the *MER* locus that confers resistance to *M. larici-populina* (Zhang et al. 2001). This locus was mapped to chromosome XIX in the poplar genome (Yin et al. 2004). Although the host resistance to rust can be resolved at the gene level, understanding the infectious behavior of rust on the leaf surface is critical in elucidating pathogen-host interactions.

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