Studies on penetration, infection and colonization of lupin roots infected by *Thielaviopsis basicola*

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ABSTRACT

The narrow-leafed lupin *Lupinus angustifolius* is highly susceptible to soil-borne pathogenic fungus *Thielaviopsis basicola* causing root rot. Light microscopy was used to study the penetration, colonization and sporulation of lupin roots by this pathogenic fungus. Events were observed in 2 to 3 days old roots produced on moist filter paper. By 24 h post inoculation (PI), infection hyphae had entered into the root hair and continued the elongation of hyphae. By 48 h PI, intercellular hyphae were present in the cortical region. By 72 h PI, intracellular hyphae were present in necrotic cortical cells. Germination of conidia within the cortical cell surrounded by host cytoplasm was observed at 72 h PI which indicates presence of secondary infection.

Key words: Light microscopy, *Lupinus angustifolius*, *Thielaviopsis basicola*.

INTRODUCTION

The soil-borne fungal pathogen *Thielaviopsis basicola* (Berk. & Broome) Ferraris (synanamorph *Chalara elegans* Nag Raj & Kendrick) is causing black root disease in numerous plant species. This fungus is worldwide distributed and commonly found in cultivated and non-cultivated soils (Yarwood 1974; Yarwood 1981). Host plants are ornamental as well as agricultural plants such as many legumes, e.g.: groundnut (His, 1978), bean and pea (Tabachnik *et al*., 1979), and lupin (Thalmann and Struck 2008). Other host plants are carrots (Heller, 2012), tobacco (Gayed 1972), cotton (Mathre *et al.,* 1966), citrus (Tsao and van Gundy 1962) and pansy (Mims *et al*., 2000). Infection of susceptible hosts results in black root rot symptoms such as dark cortical lesions, root pruning, and foliar stunting. A pictorial disease severity key for black root rot of pansy has been provided by Copes and Stevenson (2008). Detection and quantification of this pathogen within the soil has been described (Punja and Chittaranjan 1994, Heller 2012).

Thielaviopsis basicola has been reported to enter host roots through wounds (Punja *et al.,* 1992) and through root hairs and root epidermal tissue (Hood and Shew 1996, Jones 1991, Mathre *et al.*, 1966, Prinsloo *et al.*, 1992) as well as penetration through non root hair epidermal tissues (Mims *et al.*, 2000). Development of *T. basicola* during penetration and colonization of several hosts has been examined histologically. However, observations regarding the infection process are inconsistent. Formation of appressoria was reported on cotton (Mauk and Hine 1988), carrots (Punja *et al.*, 1992), peanuts (Jones 1991), and chicory (Prinsloo *et al.,* 1992), whereas direct penetration without appressoria was observed on beans (Christou 1962) and holly (Wick and Moore 1983). As noted by Hood and Shew (1996) there have been numerous reports of the formation of slender threadlike penetration hyphae with swollen terminal vesicles in plant root cells attacked by *T. basicola*. However, prior to their study of the infection of tobacco roots by *T. basicola*, the development of these distinctive infection structures had not been examined in any detail. By utilizing tobacco roots grown in agar in Petri plates, Hood and Shew (1997) were able to conduct a careful *in situ* time-course study of penetration and infection of tobacco root hairs by *T. basicola*. Stages in infection were observed by inverting plates on the microscope stage following spore germination (about 8 h) or as hyphae grew through the agar to contact root tissues (about 12 to 24 h).

Their use of non-destructive light microscopic observations allowed them to accurately document the timing and sequence of events that occurred from the moment the pathogen contacted a root hair until the time of infection vesicle developed within that same root hair. They also used transmission electron microscope to examine the mature infection structures of *T. basicola* but did not study the initial penetration and infection events using this technique.

Lupins belong to a highly divers and widespread genus and has been cultivated for at least 2,000 years (Gladstones 1998). They have a long tradition of use as food in Mediterranean and Andean cultures. Today they are grown as both forage and grain legume in the Russia, Poland, France, Germany, South Africa, and the Mediterranean, and as a cash crop in Australia where they are exported to the European and East Asian feed markets (Cowling *et al.,* 1998). One of the most important species is *Lupinus angustifolius*, the so-called narrow-leafed lupin. These lupins are highly susceptible to various soil-borne pathogens (Kaufmann *et al.,* 2011). In North-East Germany *T. basicola* has been isolated from roots and stem basis of narrow-leafed lupins (*L. angustifolius*) and has been identified as a pathogen with high impact on lupin production. The first typical disease symptoms are brown bands at the stem base, the root system turns black and dies; the leaves turn yellow and the affected plants appear undersized and dwarfish (Thalmann and Struck 2008).

In the current study the infection process of *Thielaviopsis basicola* in roots of *L. angustifolius* were examined light microscopically. Combined with the observation of Hood and Shew (1996 and 1997) and (Mims *et al.,* 2000) the result we report here should provide a detailed description of the way in which this important hemibiotrophic pathogen attacks on lupin roots and the way in which lupin roots respond to penetration, infection and colonization.

MATERIALS AND METHODS

1. Maintenance of pathogen and preparation of inoculum:

The isolate of *T. basicola* used in this study was obtained from an infected lupin plant in North Germany near Rostock. It was grown on carrot juice agar medium in the dark at 20°C (Thalmann and Struck 2008). The endoconidia used to inoculate lupin roots were obtained by flooding 23 days old culture in Petri plates with sterile deionised water and rubbing the agar surface with sterilised rubber policeman. The suspension was filtered through four layers of sterilised cheesecloth to remove chlamydospores and hyphal fragments. Five μl containing 10^4-10^5 conidia per ml suspension as determined with haemocytometer were taken to inoculate lupin roots.

2. Preparation of plant roots, inoculation procedures, and preparation of samples for light microscope:

Seeds of *L. angustifolius* (cv. Vitabor) were surface sterilised with 1.5% sodiumhypochloride for 1 minute. Seeds then were washed in multiple changes of sterilised deionised water, germinated on pieces of moist sterile filter paper in Petri plates and then have been kept in dark at 22 ^{o}C to 25 ^{o}C. After a total 2 to 3 days roots were usually 15 to 20 mm long. A pipette was used to apply a suspension of endoconidia along the length of each root, and plants were kept in dark in green house. At 24 h after inoculation, a razor blade was used to cut the roots. Cotton blue was used as a stain. All events of infection were observed under light microscope. Observation concentrated on the zone of elongation between the tip of each root and the zone of maturation where numerous root hairs were present.

RESULTS AND CONCLUSION

By examining flat embedded roots with light microscopy (Fig. 1 to Fig. 4), it was possible to locate germinated conidia and penetration sites as well as fungal structures inside root cells. This approach not only was much more productive than the random sectioning of roots, it also made it possible to find and examine many different structures and sites from a large number of roots in a timely manner.

We did not observe any endoconidial germination and there was no evidence of host cell wall as well as root hair penetration at 3 h PI. Some endoconidia were lying on the surface of the root hair and on the cell wall without any sign of germination. Most endoconidia of *T. basicola* had germinated at 24 h PI. Each endoconidia produced only a single germ tube and penetrated through root hair (Fig. 1). There was no evidence of extracellular material associated with the root tips.
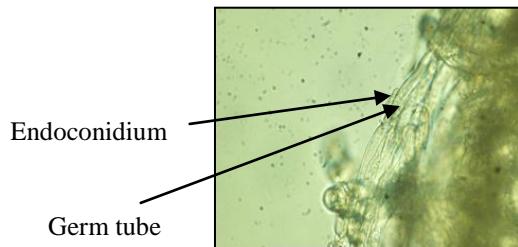
Fig.1. Infection of lupin root hair by *Thielaviopsis basicola* at **24 h PI**: Germination of endoconidium and formation of germ tube through the root surface.
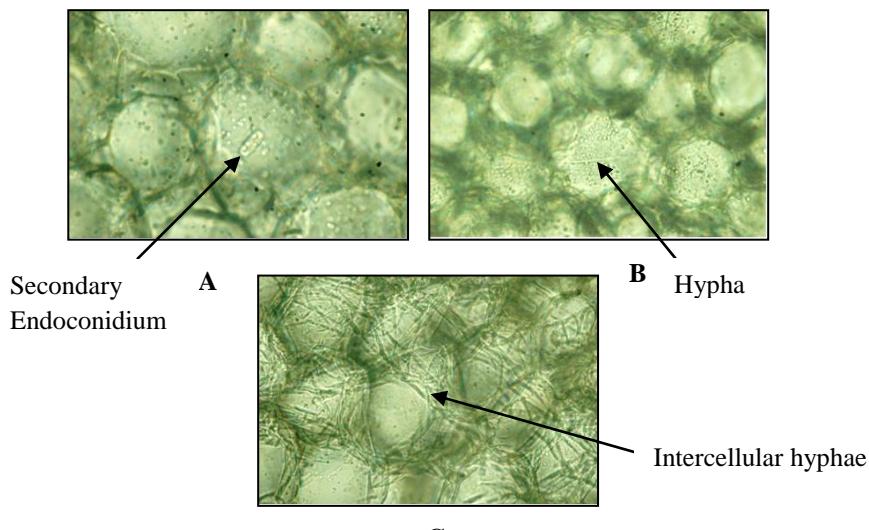
Fig.2. *Thielaviopsis basicola* at **48 h PI**. **A**; Secondary endoconidium in the cortical cell surrounded by host cytoplasm. **B,** Growth of hypha in cortical cell surrounded by host cell cytoplasm. **C**, Growth of intercellular hyphae in the cortical tissues.
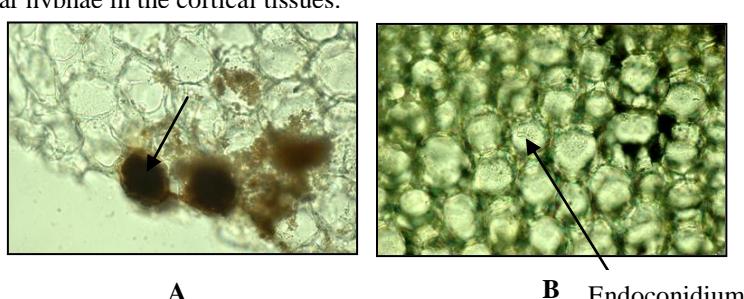
Fig.3. *Thielaviopsis basicola* after **72 h PI**. **A,** Brown coloured lesions (marked with arrow) indicate sites of attempted colonization.
B, Germination of endoconidia in the cortical cell.
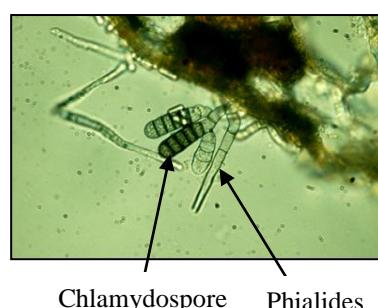
Fig.4. Sporulation by *Thielaviopsis basicola* on lupin root surface at **168 PI**. phialides and chlamydospore production on the surface of the root.

At 48 h PI many endoconidia were lying in the cortical cells surrounded by dense cytoplasm as compared to the non-infected cortical cells. Those endoconidia were secondary endoconidia (Fig. 2A). There were growths of hyphae in the cortical cells surrounded by host cytoplasm (Fig. 2B). Here at this stage we observed many changes in the structure of the host tissues because of the host pathogen. Important observation was aggregation of intercellular hyphae in the cortical tissues (Fig. 2C).

By 72 h PI, fungus started to show its necrotrophic behaviour that causes the death of host tissues. These cells developed dark brown macroscopic lesions near the root surface and growth of intracellular hyphae of *Thielaviopsis basicola* (Fig. 3A) Germination of endoconidium in the cortical cell indicates the secondary infection of *Thielaviopsis basicola* (Fig. 3B).

Sporulation was observed in colonized cortical cells at 168 h PI. Growth of phialides and characteristic chlamydosopores with dark melanized pigments were observed (Fig. 4).

The subsequent events involved the formation of penetration hypha through root hair, infection hyphae (intercellular hyphae) in the cortical tissues and sporulation. Although the majority of histological studies concerning host infection by *T. basicola* has centered on colonization and lesion development within the root cortex, root hairs have been repeatedly noted as infection sites (Christou 1962, Hood, and Shew 1996, Mathre *et al.*, 1966, Prinsloo *et al.*, 1992) and often may be the host tissue with which *T. basicola* makes initial contact.

Upon penetration of root hairs, *T. basicola* formed the threadlike penetration hyphae. As we did not observed for lupin roots, Hood and Shew (1997) reported that many germ tubes of *T. basicola* continued to elongate after making contact with root hairs. The important thing we observed that growth of both intercellular and intracellular hyphae were present in the cortex region and three days after inoculation, macroscopic symptoms were observed on root. Cells developed brown coloured lesions indicating cortical necrosis. Germination of endoconidia within the cortical cell was observed at 72 h PI which indicates the secondary infection. Sporulation among the necrotic cells indicated that host cell necrosis was not inhibitory to normal growth and reproduction of the fungus.

Growth of both intercellular and intracellular hyphae and germination of endoconidia within the cortical cell were the important observations of our study.

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