Distribution and morphology of *Neotyphodium* endophytes in apical meristems of host plants as observed using differential interference contrast microscopy.

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Abstract

Seed transmitted *Neotyphodium* endophytes colonise the apical meristem (growing point) of young seedlings and later spread into mature leaves and flowers of host plants. However, their location and orientation in the meristems, and the process of their spread into other parts of the plant, are not fully understood. To elucidate this process, the morphology and orientation of mycelia of three *Neotyphodium* endophytes (*N. coenophialum, N. lolii* and *N. occultans*) and their sexual relative, *Epichloë typhina* were observed using dissected meristem tissues with differential interference contrast microscopy, after chemical clearing. Mycelia of *Neotyphodium* species more or less ramified in pith or nodal tissue beneath the apical meristem, but were sparsely branched within apical meristems and inflorescences developing from the zone. The distribution of *E. typhina* mycelia was similar to the *Neotyphodium* species, but they sometimes appeared on the surface of the merstematic tissues.
Keywords: apical meristem, differential interference contrast microscopy, hyphal growth

Introduction

Neotyphodium Glenn, Bacon & Hanlin species are asexual fungal symbionts (endophytes) of cool-season grasses and are closely related to the choke pathogen *Epichloë* (Fries) L.R. & C. Tulasne species. Both the symbionts and the pathogen have important roles in the natural and agricultural ecosystems (reviewed in Clay & Schardl 2002). Considering that the sole confirmed transmission method of *Neotyphodium* endophytes is by infection of embryos in inflorescences of host plants (i.e. vertical transmission; Clay & Schardl 2002), understanding of the growth of endophytes in apical meristems, which later develop into leaves and inflorescences, is important. To our surprise, and contrary to the increasing interests in their growth regulation within host plants (Christensen *et al.* 2001, 2002; Tan *et al.* 2001; Tanaka *et al.* 2006; Zhang *et al.* 2006), there are only limited references concerning observation of these endophytes/pathogens in apical meristems (Christensen & Bennett 2005; Philipson & Christey 1986). To elucidate our lack of knowledge, endophyte morphology and orientation were observed using a method modified from Sugawara *et al.* (2004), which successfully visualised their mycelia within host ovules.

Methods

Tall fescue (*Festuca arundinacea* Schreb. cv. SR8200), perennial ryegrass (*Lolium perenne* L. cv. Brightstar), and Italian ryegrass (*L. multiflorum* Lam. clone Fu4-A3 and C5) infected with *Neotyphodium* endophyes were used in this study. Seeds of tall fescue and perennial ryegrass cultivars were purchased from local seed suppliers. The presence of the endophyte within those two cultivars was reported in DelCurto (2000) and Rose-Fricker & Meyer (1995). With current nomenclature, those endophytic fungi are *N. coenophialum* [Morgan-Jones & Gams] Glenn, Bacon, & Hanlin and *N. lolii* (Latch, Christensen & Samuels) Glenn, Bacon & Hanlin respectively. Italian ryegrass clone Fu4-A3 is an open pollination offspring of clone no. 54-10-4-4 (Sugawara *et al*. 2006), and clone C5 was collected from a naturalised population in Hyogo prefecture, Japan. Both clones were infected with *N. occultans* C.D. Moon, B. Scott & M.J. Christensen, as determined by microscopic observations of the plants and/or DNA analysis of its seed parent (Sugawara *et al*. 2006). These grasses were grown in a glasshouse at the National Institute of Livestock and Grassland Science (NILGS), Nasushiobara, Tochigi, Japan. Timothy (*Phleum pratense* L.) infected with *Epichloë typhina* (Pers. ex Fr.) Tul. clone T34 was also used in this study. The clone had been maintained vegetatively in the glasshouse as reference plant material which expresses choke symptoms every spring. The infection status of the grass clones was confirmed microscopically by observing the inner cortex of leaf sheaths and/or hand sectioned basal parts of the tiller stained with lactic acid containing 0.1% (w/v) acid fuchsin (Ohkubo *et al.* 2000).

To observe mycelia in apical meristems, tillers of the different grass/endophyte associations were collected between spring and summer in 2006. They were dissected under a dissecting microscope to isolate apical mersitems, and these were mounted on glass slides with a solution made by mixing lactic acid, glycerol, and distilled water in the ratio of 1:2:1 (v/v/v) (lactic acid in glycerol; Kirk *et al.* 2001), as shown in Figure 1. Mounted samples were kept at room temperature and observed periodically for several days with a differential interference contrast (DIC) microscope (Olympus BX50 with interference contrast filters; Olympus,Tokyo, Japan), up until the host tissues became clear enough for mycelia to be seen. Photographs were taken with the cleared host tissues under the above mentioned DIC microscope to record the plant morphology and morphology and orientation of the mycelia. For repeating observations and comparison, the slides were kept in a refrigerator at 5ºC to prevent too much denaturalisation of the tissues.

Results and Discussion

The method visualised mycelia of those fungi in the meristems (Figs. 2-5). Mycelia were more or less ramified throughout pith or nodal tissue beneath the apical meristems (Figs. 2 & 3), but were sparsely branched within apical meristems (Figs. 2 & 3), and young inflorescences (Fig. 4). The distribution of *E. typhina* mycelia was similar to that of the *Neotyphodium* species but some mycelia were present on the surface of the meristematic tissues, evidence of their pathogenicity which later chokes the entire inflorescence (Fig. 5). These observations correspond with the results from transmission electron microscope (TEM) observations (Philipson & Christey 1986; Christensen & Bennett 2005), but endophyte mycelia they observed in leaf primordiums were not seen in our observation. The mycelia are either sparse in the tissue, or they are thin and embedded tightly within small inter cellular spaces there, which makes recognition through

Figure 1 Protocol for apical meristem observation of grass plants with chemical clearing and differential interference contrast (DIC) microscopy. 1) Hand section a grass tiller at the point a little above the apical meristem, 2) Dissect the tiller and make a small section containing the apical meristem, 3) Mount the section with lactic acid in glycerol and observe with a DIC microscope.

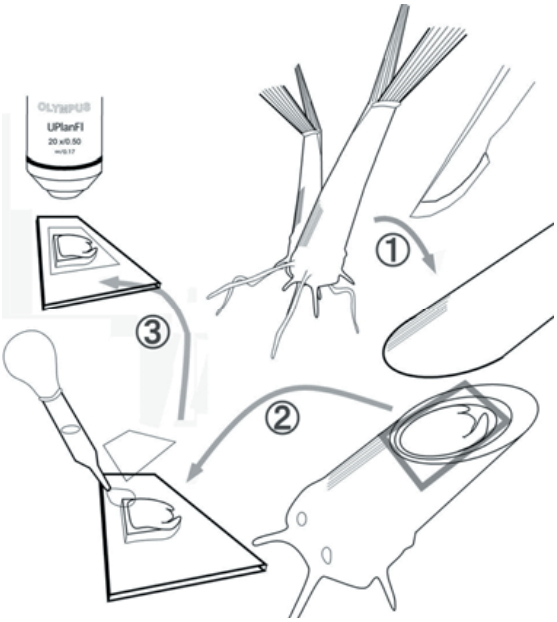


Figure 3 *Neotyphodium* endophyte in an apical meristem of perennial ryegrass (*Lolium perenne* L.) observed with differential interference contrast microscopy after chemical clearing of the tissue. A, apical meristem; L, leaf primordium; M, mycelium of endophyte. Bar: 50 µm.

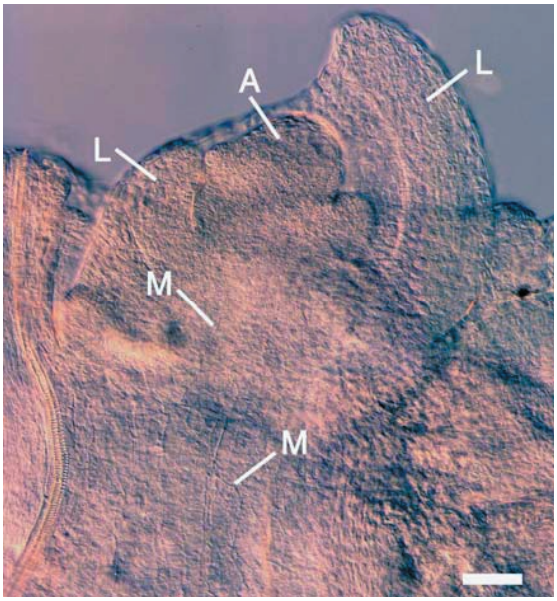


Figure 2 *Neotyphodium* endophyte in an apical meristem of tall fescue (*Festuca arundinacea* Schreb.) observed with differential interference contrast microscopy after chemical clearing of the tissue. A, apical meristem; L, leaf primordium; M, mycelium of endophyte. Bar: 50 µm.

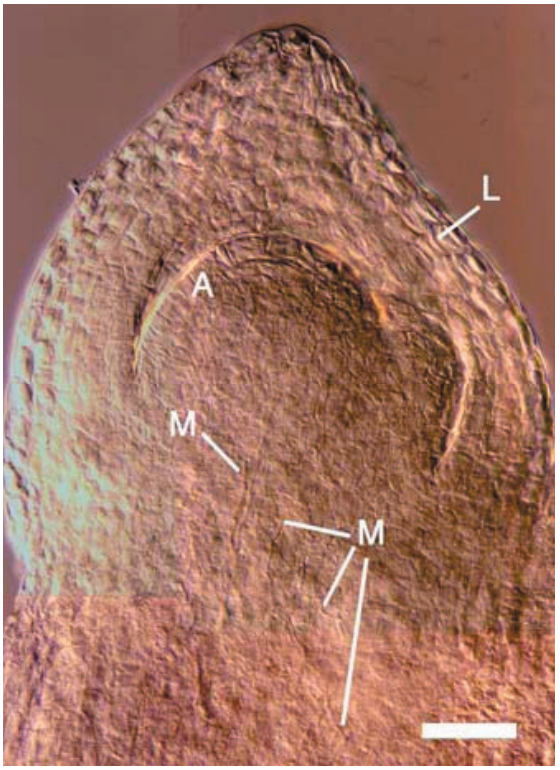


Figure 4 *Neotyphodium* endophyte in developing inflorescences of Italian ryegrass (*L. multiflorum* Lam.) observed with differential interference contrast microscopy after chemical clearing of the tissue. 1) An inflorescence in very early developing stage (clone Fu4-A3); 2) A slightly older inflorescence (clone C5), M: mycelia of endophyte, Bars: 50 µm.

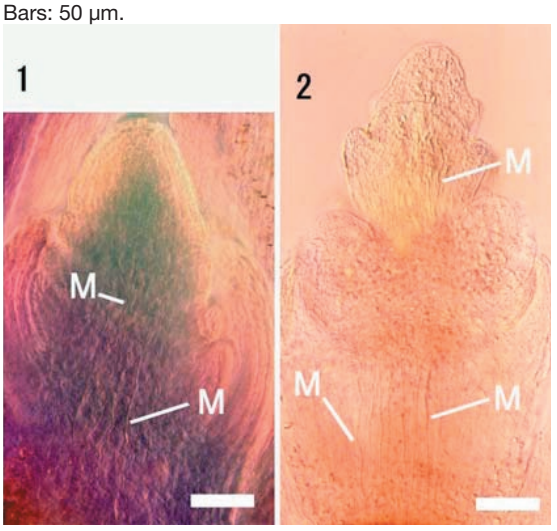
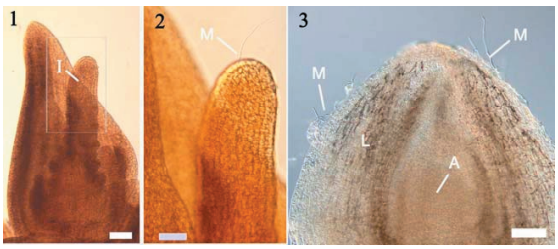


Figure 5 *Epichloë typhina* observed around apical meristem/young inflorescence of infected timothy (*Phleum pratense* L.). Differential interference contrast microscopy with chemical clearing of the tissue. 1) A young inflorescence of timothy; 2) Close-up view of the area in the gray rectangle in "1"; 3) An apical meristem covered with leaf primordiums, A: apical meristem, I: young inflorescence, L: leaf primordium, M: mycelium of E. typhina ; Bars: 50 μm.



light microscopy, including DIC, difficult. However, our method clearly visualised morphological translation of the mycelia, ramified growth seen in pith and nodal tissue, to sparsely branched, straight forward (or stretched?) growth within apical meristems and developing inflorescences (Figs. 3 & 4), which cannot be observed with TEM.

Chemical clearing of plant tissues has been widely used for microscopic observations of their morphologies (Herr 1971; Yadegari *et al*. 1994), but it has not been well applied to plant tissues infected with endophyte. Although the method we used here requires the use of DIC microscopy, it involves only simple, non-toxic chemicals and is easy to perform. Combined with other observational methods such as TEM, the method we report here will give some new insights into grass-fungi interactions.

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