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Commentary

**Endophytic coming out: the expressorium as a novel fungal structure specialized in outward-directed penetration of the leaf cuticle**

Plants develop beneficial interactions with several microbes, including bacteria and fungi. The diversity of such associations is not restricted to the taxonomical status of the interacting microorganisms, but extends to the features of their accommodation by the host and the consequent type of symbiotic relationship, ranging from optional advantages to full dependency. Within such broad – and often faded – boundaries, the detailed study of single plant–microbe associations may reveal the unexpected. This is the case with the paper Becker *et al.* present in this issue of *New Phytologist* (pp. 000–000), where an elegant approach combining confocal and electron microscopy observations with the use of mutant strains depicts a novel type of specialized hyphal structure – a so-called expressorium – in the grass endophyte *Epichloë festucae* (Fig. 1).

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*‘Surprisingly, the fungus does not leave the mesophyll through stomata – a route that several leaf pathogens use in both directions – but rather opens its way across the epidermis and cuticle.’*

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Unlike other species from the same genus, *E. festucae* provides its host with increased tolerance to biotic and abiotic stresses. This is not the only unusual feature in this *Lolium–Epichloë* association. In order to reach the leaf mesophyll, the fungus resides in the shoot apical meristem and enters leaf buds as they develop on its sides. *Lolium perenne* leaves develop by extensive intercalary growth, and the mesophyll-dwelling endophyte has to keep the pace: reducing hyphal tip growth to the advantage of intercalary proliferation is the strategy adopted by *E. festucae*. However, as soon as leaf development slows down, *E. festucae* switches back to tip growth, develops between epidermal cells and pierces the cuticle to reach the phylloplane. Here the fungus develops an extensive hyphal network that eventually harbours conidiophores.

The detailed microscopical analyses performed by Becker *et al.* reveals several intriguing traits. First of all, the epicuticular mycelium displays a high frequency of anastomosis. Such hyphal fusions are often related to a more efficient distribution of nutrients or signals across the mycelial network, or interpreted as a hallmark of functional specialization (Read *et al.*, 2009). Leaf-infecting species of *Neurospora*, *Colletotrichum*, *Botrytis*, among others (Roca *et al.*, 2005), are known to develop anastomoses between conidial germ tubes, as a prerequisite to host infection. But the case of *E. festucae* looks almost reversed: here the anastomosing hyphae have already breached the cuticle from the inside and are not going to penetrate it again. Such a developmental strategy – which goes alongside an important change in cell wall chitin distribution – could be a response to the harsher phylloplane conditions compared to the protected endophytic niche. The extensive growth of epiphyllous mycelium could, in addition, play a role in protecting the host from potential pathogens through niche competition, but also limiting leaf attractiveness for herbivores (Niones & Takemoto, 2014).

Becker *et al.*'s most impressive observation is anyway related to the process used by *E. festucae* to reach the phylloplane. Surprisingly, the fungus does not leave the mesophyll through stomata – a route that several leaf pathogens use in both directions – but rather opens its way across the epidermis and cuticle. It is reasonable to speculate that hyphal passage would affect stomatal functionality and ultimately the host physiology and fitness – a drawback that selective pressure may have discouraged in a beneficial endophytic interaction. Instead, hyphal tips grow outwards between adjoining epidermal cells, then switch to depolarized growth and swell as they reach the base of the cuticle; eventually, polar growth is re-established and a new hyphal tip penetrates the cuticle, reaching the open air. The resulting swollen hyphae are recognized by Becker and colleagues as novel structures, specialized in outward-directed cuticle breaching and appropriately called expressoria (Fig. 1), to underline their partial similarity with inward-directed, cuticle breaching appressoria, typical of most leaf pathogens.

By comparing the expressoria with other hyphal types that are specialized in the penetration of resistant surfaces – appressoria and hyphopodia – we can

depict a correlation between the resistance of the attacked surface and fungal cell specialization and complexity. At one end of this range is the appressorium, the most powerful perforating fungal structure: this dome-shaped, thick-walled, highly [melanised](#) hyphal compartment develops from conidial germ tubes or vegetative hyphae of pathogenic fungi and oomycetes, on the surface of plant aerial organs (Wilson & Talbot, 2009). Appressoria generate impressive turgor pressure by accumulating glycerol and other osmolytes [in a septum-confined hyphal structure](#). This pressure provides mechanical energy for the penetration peg to breach the cuticle and epidermal cell wall, which in the meantime is often reinforced and thickened as a primary plant defense response.

At the opposite end of the range, we can place hyphopodia (Sukno *et al.*, 2008; Bonfante & Genre, 2010; Marcel *et al.*, 2010). These swollen hyphae appear less complex than appressoria, morphologically more similar to ordinary hyphal tip compartments and normally not [melanised nor requiring a septum at the back](#). In analogy to appressoria, hyphopodia produce penetrating hyphae that head for the epidermal cell lumen and inner tissues. Nevertheless, they typically develop on cuticle-free epidermis, most often on roots, and the mechanical resistance to fungal penetration is only provided by the cell wall. Hyphopodia are common to root-interacting pathogens such as *Magnaporthe oryzae*, *Fusarium spp.* or *Colletotrichum spp.*, and symbionts such as arbuscular mycorrhizal glomeromycetes.

Expressoria represent an intermediate condition, where hyphal morphology is altered by tip swelling and repolarization, but in the absence of [melanisation](#) (and the related peak in turgor pressure). In fact, expressoria attempt the perforation of cuticle, which is more resistant than the naked cell wall, but easier to pierce than the combination of both (Dominguez *et al.*, 2011).

Beside their functional analogy, a developmental cue appears to play a conserved role in the three hyphal types: cutin monomers, in fact, have been proposed to trigger the modifications in hyphal tip growth that are associated with each of them (Wang *et al.*, 2012). Cutin is the major component of leaf cuticle, and its recognition is known to trigger appressorium development and

tip growth reactivation. The same mechanism is proposed to act in expressoria, and accumulating evidence suggests that hyphopodium development in arbuscular mycorrhizal fungi might also depend on the perception of cutin monomer mediated signals on the root surface ([Wang et al., 2012](#)).

At the end of its vegetative cycle, *E. festucae* endophytic hyphae also reach the developing flowers and colonize the ovary. Dispersal of fungal propagules within the seed grants the endophyte vertical transmission to the next generation of host plants. An alternative propagation strategy involves the horizontal transmission of ascospores and their germination on developing *L. perenne* flowers; hyphae target the ovaries to start a new vertical transmission cycle. Since *E. festucae* is not known to develop appressoria during its vertical transmission cycle, it will be interesting to investigate if such penetration structures are formed following ascospore germination during horizontal transmission.

By using mutant strains defective in catalytic (NoxA and NoxB) or regulatory subunits (NoxR), the Becker *et al.* convincingly demonstrated that Nox (NADPH oxidases) complexes are required for expressorium differentiation. Ten years ago Barry Scott's group highlighted the importance of these reactive oxygen species (ROS)-producing systems in the control of the *L. perenne*–*E. festucae* mutualistic association (Takemoto *et al.*, 2006; Tanaka *et al.*, 2006) and in particular the endophytic phase. These findings were followed by other works showing the key role of *Nox* genes in controlling basic morphogenetic processes in fungi such as apical dominance (Semighini & Harris, 2008) as well as regulating pathogenicity in plant-infecting fungi (Egan *et al.*, 2007; Giesbert *et al.*, 2008; Tudzynski *et al.*, 2012).

*E. festucae*  $\Delta nox$  mutants show extensive subcuticular growth compared to the wild type and, although still able to form hyphal swellings, they fail to polarize at the point of contact with the cuticle (Fig. 1). Nox-mediated ROS production therefore appears to be also required for the polarized growth of expressoria. Interestingly, localized accumulation of ROS is also a well known requirement for polarized growth in a few plant cell types, such as pollen tubes (Kaya *et al.*, 2014). This reinforces the concept of a conserved function for ROS as

signalling molecules in cellular morphogenetic programs across eukaryotic kingdoms.

Furthermore, appressorium development in *M. oryzae* was shown to rely on the sequential action of the two Nox complexes (Ryder *et al.*, 2013): this outlines a possible parallelism between the roles of *Nox* genes in appressoria and expressoria. It would be interesting to dissect expressorium differentiation to verify the occurrence of cytoskeleton reorientation in analogy to what has been described during *M. oryzae* appressorium formation (Ryder *et al.*, 2013).

Another interesting feature [highlighted by Becker and colleagues](#) is that [fungal cell wall composition](#) also depends on *Nox* genes: the wild type strain shows an apparent absence of chitin in endophytic hyphae (with the exception of septa) whereas the entire cell wall of  $\Delta noxA$  mutant endophytic hyphae is positive to wheat germ agglutinin-based chitin labelling. It remains unclear whether chitin is really missing or – in a more intriguing scenario – masked by the action of chitin-binding fungal effectors, as described in a few plant pathogens (Lo Presti *et al.*, 2015). This would also explain the absence of host defence responses during expressorium formation by the wild type strain, while plant cell wall thickening and callose deposition is observed in the presence of  $\Delta nox$  mutants. But the reasons for the elicitation of such a defence response may be more complex and linked to the altered physiology of the  $\Delta noxA$  mutant which shows, indeed, an increased expression of genes encoding cell wall-degrading enzymes (Eaton *et al.*, 2015).  $\Delta nox$  mutants seem, therefore, instrumental to decipher the complex interaction underneath this intimate and mutualistic association.

Although other leaf-colonizing fungi emerge on the leaf surface before sporulation, structures that resemble expressoria have never been described before. While it is possible that *E. festucae* has evolved a unique hyphal type for this scope, the impressive technical effort that was required to unveil expressorium existence, precise shape and developmental process (coupling TEM imaging of serial sections to high resolution 3D confocal imaging) suggests that analogous structures could indeed be produced by other endophytes or pathogens, but simply have not been recognized so far.

Hetherington, Nichola 8/4/2016 12:33

**Comment [1]:** Insert citation

Andrea Genre 8/4/2016 14:27

**Comment [2]:** This is described in the paper by Becker et al, object of the commentary: we have made this clearer at the beginning of this paragraph

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**Key words:** endophytes, expressorium, *Nox* genes, penetration structures, phylloplane, plant–microbe interactions.

**Fig. 1** Proposed scheme of *Epichloë festucae* expressorium development in the *Lolium perenne* leaf. An endophytic hypha (blue) develops vertically between epidermal cells and eventually produces a swollen structure under the leaf cuticle: the expressorium (left). In wild-type *E. festucae* (right, top), the combined action of mechanical force, enzyme activity (yellow) and tip growth repolarization (black arrow) results in cuticle perforation and epiphytic hyphal development. By contrast, fungal mutants for *Nox* genes (right, bottom) display an inability to restart polar growth in the expressorium and extensive subcuticular growth. This figure was kindly provided by Barry Scott.

