Secret lifestyles of *Neurospora crassa*

Hsiao-Che Kuo¹, Sun Hui¹, Jaeyoung Choi², Frederick O. Asiegbu¹, Jari P. T. Valkonen² & Yong-Hwan Lee¹²³

¹Department of Forest Sciences, University of Helsinki, Helsinki, Finland, ²Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland, ³Department of Agricultural Biotechnology, Center for Fungal Pathogenesis, and Center for Fungal Genetic Resources, Seoul National University, Seoul 151-921, Korea.

*Neurospora crassa* has a long history as an excellent model for genetic, cellular, and biochemical research. Although this fungus is known as a saprotroph, it normally appears on burned vegetations or trees after forest fires. However, due to a lack of experimental evidence, the nature of its association with living plants remains enigmatic. Here we report that Scots pine (*Pinus sylvestris*) is a host plant for *N. crassa*. The endophytic lifestyle of *N. crassa* was found in its interaction with Scots pine. Moreover, the fungus can switch to a pathogenic state when its balanced interaction with the host is disrupted. Our data reveal previously unknown lifestyles of *N. crassa*, which are likely controlled by both environmental and host factors. Switching among the endophytic, pathogenic, and saprotrophic lifestyles confers upon fungi phenotypic plasticity in adapting to changing environments and drives the evolution of fungi and associated plants.

The filamentous fungal species, *Neurospora crassa* has become a popular experimental model microbe for genetic, cellular, and biochemical research in the latter half of the 20th century¹–³. *N. crassa* is commonly found on carbohydrate-rich foodstuffs and residues of sugar-cane processing⁴. Most *N. crassa* strains collected for studies of geographical distribution have been from tropical and subtropical regions⁵. However, *N. crassa* and *N. discreta* can also be found as far north as Montana and Alaska, respectively⁶, a natural habitat that contains coniferous trees. Although the lifestyle of *N. crassa* in the wild is unknown, it normally appears on burned vegetations or trees after forest fires⁵–⁶. It has been suggested that heat from forest fires stimulates the germination of ascospores in the soil and provides a sterile, nutrient-containing environment that stimulates growth⁷. *Neurospora* species has also been isolated from the artificial plantation of *Acer ginnala* (Amur Maple) in northeastern China and proposed to be an endophyte⁸; however, no strong experimental evidence of its association with living plants is available. Global distribution and comprehensive collection of isolates of *Neurospora* species offer a new platform to decipher its ecology and evolution in nature⁹–¹². Fungi have been traditionally categorized as parasites, symbionts, or saprotrophs based on their strategies for nutrient acquisition. However, accumulated evidence suggests that fungal lifestyles are plastic in relation to their hosts and environments, rather than rigidly dictated only by their genetic makeup⁹–¹¹. Fungal endophytes exhibit a broad range of lifestyles (e.g., latent saprotrophs or pathogens, mutualists), which are determined by the fitness benefits conferred on their hosts, the production of secondary metabolites, and/or their colonization strategies¹²–¹⁴. Mycorrhizal fungi can also act as endophytes, necrotrophic pathogens, and antagonists of host or non-host plants¹⁵–¹⁷. Here, we performed a series of experiments to reveal the alternative lifestyles of *N. crassa* and provide the first evidence of endophytic and pathogenic *N. crassa* lifestyles in Scots pine (*Pinus sylvestris*).

**Results**

**Association of *N. crassa* with Scots pine.** In order to investigate the alternative lifestyles of *N. crassa*, Scots pine seedlings grown in microcosm were inoculated with conidial suspension (10⁵ conidia/ml) and the colonization patterns were documented over a period of 5 months by fluorescence and confocal microscopy (Supplementary Fig. S1). Most of seedlings looked healthy and were indistinguishable from those without inoculation. Surprisingly, however, fungal hyphae expressing GFP were observed from inside of inoculated seedlings, but not from uninoculated ones. During its growth in the cells of Scots pine seedlings, *N. crassa* was found to proliferate and survive for up to 5 months without causing any disease symptoms, suggesting its endophytic lifestyle. Inside the roots, fungal growth was confined mostly to the root epidermis and cortex layers (Supplementary Fig. S1e). More compelling evidence of endophytic lifestyle is being described in the later section. To further decipher the innate association between *N. crassa* and Scots pine, we performed a series of inoculation experiments on Scots pine seedlings grown on water agar.
Can *N. crassa* be a plant pathogen on Scots pine? Our most remarkable finding is that *N. crassa* can act not only as an endophyte but also a pathogen on Scots pine, when the host plant was grown on water agar or under controlled environments in the greenhouse. Infection with *N. crassa* incited typical disease symptoms, eventually causing the death of Scots pine seedlings. The mortality rate reached to 83% (90 out of 108 seedlings) at 5 weeks post inoculation (wpi) (Fig. 1a). The abiosis of Scots pine caused by *N. crassa* takes 4–5 weeks, whereas its well-adapted fungal pathogen *Heterobasidion annosum* exerts a similar effect in only 3–4 weeks (96% mortality rate) (Fig. 1b). During the initial stage of infection, *N. crassa* conidia germinated, formed a hyphopodia-like structure (Supplementary Fig. S2c), penetrated into plant tissues, and grew intra- or intercellularly between adjacent cells (Fig. 1d–i; Supplementary Fig. S2d–f; Supplementary Fig. S3). Invasive growth continued from root cortical cells (Fig. 1d–e) to the core area (Fig. 1f), and they were found in almost 50% of infected root cells at 5 wpi (Supplementary Fig. S4). At the end of the infection stage, *N. crassa* hyphae could grow out from the stomata on the stem of infected Scots pine seedling (Supplementary Fig. S5a–f) and develop conidiophores with conidia (Supplementary Fig. S5g). These observations clearly demonstrate that *N. crassa* can complete its life cycle in association with Scots pine and further support the hypothesis that *N. crassa* has a pathogenic lifestyle. Moreover, culture filtrate of *N. crassa* induced similar cell death in Scots pine seedlings (Supplementary Fig. S6b), suggesting that *N. crassa* may produce phytotoxic compounds and function as a necrotrophic plant pathogen on Scots pine. To understand if *N. crassa* can incite disease symptom not only in seedlings grown on water agar, but also grown trees, 3-year-old Scots pine trees were inoculated with wood dowels pre-colonized by *N. crassa* in the greenhouse. *N. crassa* could incite clear necrosis on 3-year-old trees 6 wpi. The necrosis areas by *N. crassa* and *H. annosum* were 42.5% and 67.2%, respectively, when measured by the ratio of the white (healthy) and brown area (necrosis) (Fig. 2a–c). These infected trees were sampled and heat-treated at 121°C for 10 min to understand whether *N. crassa* inside of tree could survive under harsh conditions such as forest fire. Surprisingly, *N. crassa* was grown out and was the sole surviving fungal taxon on wood trunks after heat treatment when incubated for 2 weeks (Fig. 2d). This further supports our previous finding and suggests that *N. crassa* within host cells can survive as a pathogen or an endophyte and grow out from the burned tree as a saprotroph.

**Biochemical and molecular mechanisms underlying *N. crassa* and Scots pine interactions.** To determine whether *N. crassa* infection elicits a defense response in Scots pine seedlings, host-defense-related reactions were observed. Plant cells at infection sites killed by *N. crassa* were observed by staining with Evans blue (Figure 1| Pathogenic interactions between *N. crassa* and Scots pine seedlings. Scots pine seedlings were inoculated with *N. crassa* (a), *H. annosum* (b), and control (c). (d) Transverse section of Scots pine root inoculated with *N. crassa*. Plant cell walls were stained with PI, and fungal hyphae were labeled with WGA. (e and f) Transverse sections of Scots pine roots inoculated with *N. crassa* FGSC 10589. GFP images were obtained by staining with FM4-64 at different stages of infection from 3 (e) to 5 (f) weeks post inoculation (wpi). (g) Image of *N. crassa* hyphae stained with WGA within host plant cells. (h) SEM image of *N. crassa* hyphae growing from one plant cell to another. (i) Colored SEM image, red and green indicate plant cell wall and *N. crassa* hyphae, respectively. Bars = 1 cm (a–c); 100 µm (d, e, and f); 10 µm (g); 5 µm (h). *N. crassa* strains used in a, d, g, h, and i was FGSC 2489. **
(Supplementary Fig. S6a). Furthermore, callose deposition and the accumulation of ROS around infection sites on stem were evident following staining with aniline fluorochrome blue (Fig. 3a) and diaminobenzidine (DAB) (Fig. 3b), respectively. These data indicate that the interaction between N. crassa and Scots pine represents a typical host–pathogen interaction.

In addition to biochemical response, expression patterns of ROS-and defense-related genes, such as those encoding peroxidases\(^{15}\) (peroxidase 65 [PER65], class III peroxidase [PSYP1], and glutathione peroxidase [GPX]), Avr9/Cf-9 rapidly elicited defense-related gene (ACRE)\(^{16}\), defensin (DEF1)\(^{17}\), and catalase (CAT)\(^{15}\) were monitored by qRT-PCR. All genes were differentially expressed in Scots pine’s roots at 2 wpi with N. crassa (Fig. 3c). Expression of genes encoding catalase and peroxidases was most highly up-regulated. Expression of ACRE and DEF1 was also up-regulated in the roots (Fig. 3c) and stems (Supplementary Fig. S7a) after N. crassa infection. We also monitored the expression profiles of pathogenicity-related genes in N. crassa, including those encoding necrosis-inducing protein (nip)\(^{18}\), endoglucanase IV (egl-4)\(^{16}\), catalase (cat), peroxidase (per), and two oxidoreductases (otti-1 and oxi-2).

Expression of egl-4 was highly up-regulated in roots (Fig. 3d and Supplementary Fig. S7b). Similar expression pattern of egl-4 was observed in H. irregulare during infection of Scots pine\(^{19}\). Expression of the genes encoding the two oxidoreductases and catalase were also highly up-regulated during interaction with Scots pine seedling (Fig. 3d). Together, expressions of genes responsible for ROS modulation were highly up-regulated in both the host and the pathogen. ROS is known to play key roles in maintaining the balance between endophytic and pathogenic fungal lifestyles with host plants\(^{20}\). Reducing ROS levels in the host can stimulate latent pathogens to cause disease\(^{21}\) in the roots. Indeed, deletion mutants showed significantly lower virulence on Scots pine seedlings (\(p = 0.02\) and 0.07 in ∆otti-1 and ∆otti-2, respectively) (Supplementary Fig. S8), although they did not have any defect on mycological characters including mycelial growth, colony morphology and pigmentation. These combined biochemical and gene expression data suggest that the association between N. crassa and Scots pine is a typical intimate host–pathogen interaction.

Discussion

Understanding of lifestyle switching in fungi is critical for deciphering the evolution of host–microbe interactions and carbon/nitrogen cycling in the ecosystem. Our data and previous reports\(^{22-11}\) suggest that fungal lifestyles are not stable but dynamic, and are likely influenced by the genetic makeup of the fungal species, host factors, and changing environments. The endophytic stage represents a balanced interaction between the fungus and its host. However, endophytic fungal species can become pathogens or saprotrophs when this balance is disturbed or the host dies, respectively. In contrast,
saprotrophic wood decomposers can colonize Scots pine’s roots as mycorrhizal fungal species\(^25\). Opportunistic fungal pathogens, including *Aspergillus fumigatus*\(^26–27\) and *Candida albicans*\(^28\) are pathogenic only in immunocompromised humans\(^26–28\). Therefore, many fungi have likely evolved to switch their lifestyles among the Endophyte–Pathogen–Saprotroph as a circle (EPS Ring; Fig. 4) to adapt to various hosts and changing environmental conditions. However, the mechanisms underlying the appearance of *N. crassa* as the first fungal colonizer after forest fires in nature remain unknown. Although ascospores rather than conidia on the soil or on the tree were proposed as a source of *Neurospora* after a forest fire\(^6,29\), this is contradicted by the fact that conidia are observed most frequently in the field\(^5\). It has also been reported that desiccated conidia can survive after treatment at 100°C\(^30\) and our data also provides the evidence that *N. crassa* can survive on wood trunks after heat-treated (Fig. 2d). However, we do not rule out the possibility that ascospores could also survive such extreme temperature. These further suggest that *N. crassa* within host cells can survive and grow out from the burned tree in which it resides as a pathogen or an endophyte. To better understand the ecology of *N. crassa* and provide more ecological relevance of our findings, we attempted to detect *N. crassa* from forest soil samples collected from tropical (Indonesia) to Nordic (Finland) areas including post-forest fire sites (Supplementary Table S1). We were unable to amplify *N. crassa* ITS region by PCR from these soils. Even when soil DNA samples from post-forest fire sites were analyzed by pyrosequencing, no sequence signature was found as *Neurospora* species. These results would support our hypothesis that *N. crassa* may not be living as a saprotroph in forest soils but as an endophyte or a pathogen in their natural host, Scots pine. Taken together, our study will provide a new paradigm to understand fungal lifestyles in nature and coevolution with its associated plants.

### Methods

**Plant and fungal materials.** *Neurospora crassa* strains (Supplementary Table S2) used in this study were obtained from the Fungal Genetic Stock Center, Missouri, USA, and grown on Vogel’s medium (pH 6.5)\(^2\). *Heterobasidion annosum* P-type (isolate 03012 provided by Kari Korhonen, METLA, Finland) was maintained on MEG medium (0.5% malt extract, 0.5% glucose, 2% agar). Scots pine (*Pinus sylvestris* L.) seeds were obtained from Svenska Skogsplantor (Saleby FP-45), Finland and sown on 2% water agar plates (or test tubes) or a mixture of sterilized Kekkilä White 420-W peat for microcosm assay. Prior to use, 100 seeds were sterilized by

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**Figure 3** | Host response and gene expression during interaction with *N. crassa*. (a) Callose deposition around the infection site on stem, stained with aniline fluorochrome. (b) Accumulation of reactive oxygen species (ROS) at the infection site on stem, stained with diaminobenzidine (DAB). Bars = 20 μm. (c) Expression profiles of defense-related genes in the roots of Scots pine after *N. crassa* inoculation: ACRE, Avr9/Cf-9 rapidly elicited defense-related gene; PER65, peroxidase 65; PSYP1, class III peroxidase; GPX, glutathione peroxidase; DEF1, defense; and CAT, catalase. (d) Expression profiles of *N. crassa* genes during its interaction with Scots pine’s roots. nip, Necrosis-inducing protein; cat, catalase-1; per, dyp-type peroxidase; oxi-1 and oxi-2, two oxidoreductases. The level of expression was measured at one (T1) and two (T2) weeks after inoculation. Fold changes are relative to uninfected Scots pine seedlings (c) and *N. crassa* grown on Vogel’s medium (d). The bars indicate standard deviation among 3 biological replicates.

**Figure 4** | Dynamic relationship between a fungus and its host plant as a function of the fungal lifestyles, Endophyte–Pathogen–Saprotroph, as a circle (EPS Ring). The endophytic stage represents a balanced interaction between fungal virulence and host defense factors. When this balance is disturbed or the host dies, endophytes may become pathogens or saprotrophs, respectively. Saprotrophs and pathogens may switch their lifestyles to endophytes/pathogens and endophytes/saprotrophs, respectively, in the presence of appropriate environmental factors. H, host; E, endophyte; P, pathogen; S, saprotroph.
soaking in 30 ml of 33% Hydrogen peroxide for 15 min, and then washing with 2 L sterilized water. Experiments on water agar or in microcosm were performed in growth chamber or in plant growth room, respectively. The controlled conditions, respectively, by fluorescence and confocal microscopy. To make fungal infections in the plant growth room. The fungal colonization patterns were monitored over a period of 5 weeks and 5 months for the samples on water agar and in microcosm, respectively, by fluorescence and confocal microscopy. To make fungal infections in fully developed trees, 3-year-old Scots pines were used. The trees were obtained from the field in Ruotsitsinly (field station of the Finnish Forest Research Institute, Finland) and grown in a greenhouse (Kakkula INSCAN). In the growth chamber (during the day and 18°C at night). The average height and diameter of the trees were 60 cm and 15 mm, respectively. The wood dowels (autoclaved Scots pine wood, 1 cm × 1 cm diameter) were fixed with fixing solution (2% glutaraldehyde, 2% formaldehyde, 0.1% tannic acid, 4.5% sucrose in 0.1 mM CaCl2, pH 5.6; Sigma). Diaminobenzidine (DAB) was used to detect accumulated H2O2. Aniline blue staining was used to reveal callose formation. Propidium iodide (PI) and wheat germ agglutinin (WGA) from Trichium vulgaris conjugated to fluorescein isothiocyanate (FITC) were used to stain plant and fungal cell walls, respectively.

**DNA extraction and PCR from forest soil samples**. A total of 115 forest soil samples collected from tropical (Indonesia) to Nordic areas (Finland) including post-forest fire site, was obtained from the courtesy of Kajar Koster, Department of Forest Sciences, University of Helsinki. Details on collection sites and year, and soil types are described in Supplementary Table S1. Soil DNA was extracted by using cetyltrimethyl ammonium bromide (CTAB) buffer24, and used to detect N. crassa by PCR with ITS primer pair of NicF- F (AAAAACTCCCAAACAGCATG) and NicF-R (CCGCACGTATTTGGG).

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**Author contributions**
Conceived and designed the experiments: H.C.K., Y.H.L. Performed the experiments: H.C.K. Analyzed the data: H.C.K., S.H., J.C., F.O.A., J.P.T.V., Y.H.L. Wrote the paper: H.C.K., Y.H.L.

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