

Endophytic interaction of the filamentous fungus *Neurospora crassa* with the grass *Brachypodium distachyon* roots

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Abstract

Fungi, beyond their role as saprophytes, engage in intricate relationships with plants, showcasing diverse connections ranging from mutualistic to pathogenic. We describe here an endophytic interaction employing two well-established model organisms, the grass *Brachypodium distachyon* and the ascomycete *Neurospora crassa*. Although *N. crassa* has been extensively investigated under laboratory conditions, its ecological characteristics remain largely unknown. In contrast, *B. distachyon*, a sweet grass closely related to significant crops, demonstrates remarkable ecological flexibility and participates in a variety of fungal interactions, encompassing both mutualistic and harmful associations.

Through comprehensive microscopic analysis using electron, fluorescence, and confocal laser scanning microscopy, we discovered a novel endophytic interaction between *N. crassa* and *B. distachyon* roots, where fungal hyphae not only thrive in the apoplastic space and vascular bundle, but also may colonize plant root cells.

This consistent connection between the fungus and *B. distachyon* suggests a natural association of the fungus with Poaceae. This discovery opens new perspectives for understanding the fungal life cycle in nature. Moreover, we present a tool for studying plant-fungus interspecies communication, combining well-established model systems.

INTRODUCTION

As important players in the ecosystem, fungal saprophytes can also interact with plants^{1–3}. Thus, the complex interplay between the plant and fungal counterparts is highly diverse. Pathogenic fungi may develop biotrophic or hemibiotrophic lifestyles^{4,5}. Both interactions negatively affect the plant counterpart, while the fungus benefits from the infected plant nutrients. Fungi and plants benefit from mutualistic interactions, with the advantages to the plants ranging from stress resistance to macro- and micronutrient supply to the maintenance of a specific beneficial microbiome^{6–10}. In Mycorrhiza (MR), filamentous fungi invade the root system of plants and form a stable, beneficial coexistence^{11–13}. There are two basic types of mycorrhizae: ecto- and endo-MR, where the latter includes arbuscular (AMR), ericoid, and orchid types. Ecto-MR fungi colonize only the intercellular space, while endo-MR fungi also penetrate individual cells¹⁴. Glomeromycota fungi interact with the vast majority (80%) of land plants and form eponymous structures (arbuscles) in the invaded cells in AMR^{15,16}. Ericoid^{17,18} and orchid MR¹⁹ occur exclusively in plants belonging to Ericaceae and Orchidaceae, respectively. The fungal partner belongs to either asco- or basidiomycota. Fungal growth in plant cortical cells can be observed in both cases, where fungi do not grow through plasma membranes, but rather within the apoplastic space between cell walls and membranes. In temperate trees and shrubs, ecto-MR fungi form a massive fungal mantle surrounding the primary and secondary roots²⁰. In the cortex of the plants, there is an extensive fungal network called the Hartwig net, but no cell invasion can be observed. Some fungi can also develop an endophytic lifestyle, which does not negatively affect plant development, but as it does not

develop MR-characteristic features, it cannot be classified as MR as well ^{21,22}. A successful development and maintenance of an interaction strategy requires communication between both sides, regardless of whether the interaction has a positive or negative impact on the interacting organisms ²³.

A novel, stable, and basic system is presented in our research to investigate plant-fungal interactions. The system consists of two well-established model organisms: the ascomycete *N. crassa* and the grass *B. distachyon*. Despite extensive research on *N. crassa* ^{24–28}, little is known about its natural populations and ecology ^{29–31}, and this ascomycete is described as saprophytic. Wild populations have been isolated in parts of India, Pakistan, Middle Africa, South America, and the USA (Louisiana and Texas) from grass, soil, wild sugar cane, cane stubble, and wood ^{32,33}. A single publication also reported the growth of *N. crassa* in pine trees ³⁴. These results, however, are controversial and have not yet been replicated. *B. distachyon* is a sweet grass and is closely related to important crop plants like barley and wheat ^{35–37}. There is a wide distribution of this species and it is highly adaptable in terms of its ecological environment ³⁸. In addition to pathogenic interactions, *Brachypodium* is involved in mutualistic interactions as well.

Many studies examine the properties of highly specialized plant-fungus interactions, involving highly adapted organisms, making investigations and interpretation of data challenging. Due to its simplicity, our study enables us to overcome these difficulties and decipher the general properties of interspecies interactions. As both interacting partners are well-established model organisms, the system provides us with a powerful tool for genetic and cell biological studies. This is the first report showing *N. crassa* interacting with plant roots in a stable, endophytic manner.

RESULTS

We previously described a genetic network for tryptophan-dependent auxin production in the model *Neurospora crassa* ³⁹ and started to investigate the physiological function of the phytohormone in the fungus. A few years earlier a single report was published that indicated an endophytic lifestyle of *N. crassa* ³⁴, which appeared to pair well with our research on auxin production. However, despite vigorous attempts from our side, we were not able to establish an interaction between *Pinus* roots and *N. crassa* nor with roots from *Arabidopsis thaliana* or *Zea mays* (data not shown). Finally, we employed the model grass *Brachypodium distachyon*, which is believed to interact with numerous microbes present in the soil ⁴⁰. We used two different approaches to obtain fungal-plant interaction; For the first microscopic investigations, an ecoFAB device with a liquid medium was used. For longer co-cultivation petri dishes and 50 ml falcon tubes with solid VMM with reduced sugar content were prepared. Fungal colonization by *N. crassa* of *B. distachyon* followed the same pattern in all experiments: We observed a significant growth of *N. crassa* around the plant root, while the *B. distachyon* shoot did not seem to be affected (**SFig 1**). Co-culturing of *N. crassa* with *B. distachyon* led to a significant reduction in root growth after 48 hours ($p = 3.134 \times 10^{-5}$), an effect which was even stronger after 67 hours with root growth stopping after 139 hours (Fig. 1).

Using the *N. crassa* Histon1:GFP line (#9518) which allows for easy recognition of fungal hyphae, we observed extensive fungal growth around the plant root and an attachment of hyphae to the root surface. Moreover, there was a strong formation of protoperithecia in the root vicinity (**SFig 2**). Scanning electron microscopic images of infected roots substantiated our previous observations revealing an intact root epidermis with a tightly surrounding mycelial network (Fig. 2).

The strong effect of the fungus on the plant root let us further investigate the interaction of *N. crassa* and *B. distachyon*. We collected roots with an attached fungal network, which were then prepared for on-section analyzes by confocal laser scanning (CLSM, Fig. 3 and **SFig 3**) and transmission electron microscopy (TEM, Fig. 4). Intrudingly, we observed fungal hyphae within the root, not only growing in the intercellular space but also in some of the root cortex cells and inside the vascular bundle.

Cross and longitudinal sections imaged by CLSM showed overall living root tissues, mostly unimpaired by fungal invasion. However, single hyphae showed a clear growth in the apoplastic space (Fig. 3 **red arrows**), and in single root cortex cells an accumulation of fungal hyphae was visible (Fig. 3 **yellow arrows**). Those invaded cells were in most cases separated and surrounded by non-infected, living neighbor cells, however, in some cases a whole cell layer of cortical cells showed fungal accumulation in a very similar manner (Fig. 3D). We could not observe the formation of an extensive Hartwig-net-like hyphal network in any case. Paraffin sections and semi-thin resin sections similarly indicated limited invasion of the cortical tissues (**SFig. 3**). In addition, fungal hyphae were more readily detected inside the vascular bundle. In a few cases of extensive fungal growth inside the vascular cylinder, the cortical tissues appeared notably less affected. To quantify the colonization rate of the vascular bundle by *N. crassa* in different *B. distachyon* roots, serial paraffin sections through app. 1 mm long root segments collected up to 1 cm away from the root tip were evaluated and assigned a score of 0 to 4 (0 (no hypha); 4 (extensive growth of hypha)). As shown in **SFig. 4**, except for one plant, all investigated plants showed colonization of the vascular bundle. However, the extent of colonization was generally rather low with both median (0.88) and standard deviation (1.268) values below 2 (defined as the middle of the colonization scale).

TEM images provided additional detailed information about the distribution and spreading of fungal hyphae in the plant tissues (Fig. 4). In the ultrathin sections, fungal hyphae could be detected in every layer of the plant root from the epidermis to the xylem elements of the vascular bundle. As shown in Fig. 4D and F, fungal hyphae cross the plant cell wall and enter neighboring cells over the plasmodesmata. In the case of extensive growth inside the vascular bundle, plant cells seemed largely destroyed except for xylem elements with secondary cell walls (Fig. 4G-J).

Our data clearly show that *N. crassa* exhibits astonishing behavior towards *B. distachyon* with a growth pattern in the plant, which strongly resembles an endo-mycorrhiza.

DISCUSSION

Neurospora crassa is a model organism that is widely used in research, even though little is known about its natural lifestyle. Wild *Neurospora* strains are usually collected from burned vegetation, where the genus appears as the first fungal colonizer, probably germinating from single ascospores^{32,33,41}. Nevertheless, most of the strains collected belong to *N. intermedia*. Pandit and Maheshwari conducted the only ecological study on an indigenous *Neurospora* species^{42,43}. In a sugar cane field that was burned periodically, they examined a population of *N. intermedia*. Even though *Neurospora* can be cultivated from soil samples, visible and excessive growth only occurs when vegetation has been exposed to fire. The persistence of *Neurospora* mycelia or spores between two fires remains a mystery. One study suggested that the natural habitat of *N. crassa* is inside pine trees, where the fungus shows a varied lifestyle, from mutualistic to pathogenic depending on the growing conditions [34]. Despite this, there is no evidence to support the notion that *N. crassa* is associated with pine trees.

Although there is no direct evidence that *N. crassa* infects *B. distachyon* in the wild, *B. distachyon*'s geographical distribution partially overlaps that of *N. crassa* and the two species favor similar climatic conditions. Sordariales fungi other than *N. crassa* dominate the rhizosphere microbiome of *B. distachyon*⁴⁰. There are, however, records of *N. crassa* occurring on different grass species, including wild and cultivated *Saccharum*³¹, which is closely related to *B. distachyon*. Our study shows *N. crassa* developing a stable, endophytic interaction with *B. distachyon*. The fungal interaction with *B. distachyon* does not affect its fitness in the early stages of interaction, contrary to our investigation of other plant species where *N. crassa* rapidly overgrew and eliminated the plant counterpart. Although root growth was significantly inhibited, leaf and shoot development was similar to that of non-infected plants, suggesting that the water supply was normal. *N. crassa*, however, switches from a pathogenic or saprophytic lifestyle when the balance of the interaction is disturbed. Such a lifestyle versatility is characteristic of endophytic fungi^{45,46}. Nonetheless, since endophytic fungal interactions are usually beneficial to plants²¹, it remains unclear whether *N. crassa* impacts plant development in any positive way. The results of our study also hint towards *N. crassa* in the wild being associated with the Poaceae family. The collection of wild isolates from grass rhizosphere may be a promising approach to confirm this hypothesis.

Crossing experiments with *N. crassa* in the laboratory are standard, but *N. crassa*'s sexual development in nature remains an enigma. During the present study, extensive protoperithecia formation was observed on the plant roots. The high nitrogen consumption of Poaceae could explain this phenomenon by depleting nitrogen around roots, promoting early sexual development of the fungus⁴⁷⁻⁴⁹. To determine whether protoperithecia can mature in the interaction system, further studies will be conducted.

It is the first time *N. crassa* has been shown to interact endophytically with plant roots. The fungus' interaction with *B. distachyon* is stable, which may indicate that the fungus is naturally associated with Poaceae. Hence this research provides new insight into the natural life cycle of fungi to the scientific

community. A powerful tool for studying plant-fungus interspecies communication is provided by the presented system, which combines two well-established model organisms.

MATERIAL AND METHODS

STRAINS, GROWTH CONDITIONS

The following *N. crassa* strains used in this study were provided by the Fungal Genetics Stock Center (FGSC; Kansas City, MO, USA): FGSC #4200 (wild type, mat A), FGSC #9518 (*his-3+::Pccg-1-hH1+-sgfp+*, mat A). Fungi were cultivated on Vogel's minimal medium [VMM, ⁵⁰]. Strains carrying auxotrophic markers were grown on media supplemented with the required amino acids. For co-cultivation of *N. crassa* with *B. distachyon* Vogel's minimal medium with reduced sucrose content (0,2%) was used. Fungi were cultivated in a climate chamber at 25°C under long-day conditions.

Zea mays and *Brachypodium distachyon* TR7a wild type caryopses as well as *Pinus sylvestris* wild type seeds were provided by the Botanical Garden of the CAU zu Kiel (Kiel, Germany). All seeds and caryopses were surface sterilized according to Weigel and Glazebrook ⁵¹ before cultivation. In case of *B. distachyon*, the lemma and palea of the caryopses have been removed manually prior to surface sterilization. After surface sterilization, plants were vernalized for 48 h at 7°C in the dark and cultivated under controlled sterile conditions in a climate chamber (22°C, 60% humidity, 8 h light, and 16 h dark). Plates with 7–10 days old germlings were inoculated with *N. crassa*. For this, macro conidiospores were transferred from a mycelium by a loop and plated at the roots' vicinity under sterile conditions. Inoculated plants were cultivated under sterile conditions in a climate chamber (25°C, long day).

FIXATION, SECTIONING AND STAINING

For analyzes by CLSM, infected plants were immediately fixed in 4% (w/v) paraformaldehyde for 30 min in a vacuum. After 15 min the fixing solution was replaced. Subsequently, the fixed samples were washed in 100 mM Tris/HCl, pH 8.8 with 0.1% (v/v) glycerol and stored overnight at 7°C. The root part of the samples was then divided into ca. 1 cm long sections (designated as "root tip", "S1" and "S2") embedded in 4% (w/v) agarose. 60–120 µm longitudinal sections were prepared by using a Zeiss HYRAX V50 vibratome.

For paraffin embedding, 1 cm long root terminals were cut into 2 mm segments and fixed with 4% paraformaldehyde in 200 mM HEPES, pH 7.4 for several days. Samples were dehydrated by a rising ethanol series (50-70-80-90-96-100-100%), followed by 2 steps of 100% acetone. For clearing, samples were incubated in acetone and xylol 1:1 mixture, and three steps of 100% xylol, including one overnight. Next, samples were incubated in Paraplast Plus at 60 °C overnight and embedded the next day. Blocks were cut into 7 µm sections using a Leica RM2200 rotary microtome, stretched on a warm water bath, transferred onto adhesion slides Superfrost® Plus, and stained with Hematoxylin and Eosin using a standard protocol.

MICROSCOPY

Diverse equipment was used for microscopic analyzes.

For the first analyzes of the interaction, an epi-fluorescence microscope (ECLIPSE Ci, Nikon, Tokyo, Japan) equipped with a GigE camera (Imaging Source, Bremen, Germany) was used. Epi-fluorescence filter block GFP (longpass, excitation 480/40 nm, dichroic mirror 505 nm, barrier filter 510 nm) was used for green fluorescence detection. Images were analyzed using Nikon software NIS Elements D basic.

Confocal fluorescence analyzes were performed using the confocal laser scanning microscopes Leica TCS SP5 or -especially for overview images- Zeiss LSM 900. Fusion constructs with eGFP were excited at 488 nm, and emission was detected at 500–550 nm. Images were analyzed with the Leica LAS AF Lite or Zeiss ZEN3.2 blue edition software respectively.

Stained paraffin sections were imaged with a Zeiss Primostar upright microscope equipped with an Axiocam 105 color camera and the ZEN 3.2 software, using 10x and 40x Plan-Achromat objectives.

ELECTRON MICROSCOPY

For electron microscopic analysis, 1-cm long root terminals were cut into 2 mm segments and fixed with 1% glutaraldehyde in 200 mM HEPES, pH 7.4 for several days. For scanning electron microscopy (SEM), segments were post-fixed with 1% osmium tetroxide for 30 min, and stained with 2% uranyl acetate for 1 h, dehydrated with a rising ethanol series (50-70-80-90-96-100-100%), followed by critical-point drying. Dried specimens were mounted on adhesive carbon tape and sputter coated with 10 nm Au using Bal-Tec SCD 050. Samples were imaged with a Zeiss Sigma 300 VP using 1 kV accelerating voltage and a secondary electron detector. For transmission electron microscopy (TEM), fixed samples were cut into 100- μ m thick sections using a vibratome Zeiss Hyrax V50. Sections were post-fixed with 1% osmium tetroxide for 1 hour, stained with 2% aqueous uranyl acetate for 1h and dehydrated using a graded ethanol series (50-70-80-90-96-100-100%), each step for 15–30 min, followed by acetone (100%) 2x 30 min. Samples infiltrated with increasing concentration of epon resin or Spurr low viscosity resin (both Sigma-Aldrich) (33-66-100%) over three days. Finally, segments were flat embedded between PTFE-coated glass slides and heat polymerized at 65°C for 48 hours. After re-mounting, sections were cut using a Leica UC7 ultramicrotome and Diatome diamond knives. Semi-thin, 1- μ m epon sections were transferred onto adhesion slides Superfrost® Plus, stained with Richardson's solution (alkaline solution of azure II and *methylene blue*), and imaged with a Zeiss Primostar upright microscope equipped with an Axiocam 105 color camera and the ZEN 3.2 software, using 10x and 40x Plan-Achromat objectives. Ultra-thin, 80-nm sections were deposited on copper, slot, formvar-coated grids, and contrasted with saturated aqueous uranyl acetate for 10 min, followed by Reynolds lead citrate for 3 min. Grids were imaged with a CM10 transmission electron microscope (Philips), operated at 80 kV, and equipped with a LaB6 filament, a CCD side-mounted MegaView III camera, and iTEM software (both Olympus Soft Imaging solutions).

STATISTICAL ANALYZES

For the root growth analyzes, *B. distachyon* germlings were grown vertically on a solid medium for 7 days (growth condition s. above). After 7 days, half of the germlings were inoculated with *N. crassa* macro conidiospores. Uninoculated germlings were grown as negative control under the same conditions. Root length (mm) was determined daily for 140 h post-inoculation. Data shown in Fig. 1 represent the mean of three biological replicates. Error bars correspond to the standard deviation. Significant differences between non-treated and treated root lengths were determined by T-test analysis. Significance is indicated as * (*p < 0.1, **p < 0.05, ***p < 0.005).

For investigating the extent of the infection of the *B. distachyon* root vascular bundle by *N. crassa*, paraffin sections were prepared as described above. In total nine plants were analyzed. For each plant sample, the extent of infection was evaluated on serial paraffin sections through app. 1 mm long root segments collected up to 1 cm away from the root tip. 2–4 segments were analyzed for each plant sample. The score was assigned on a scale from 0 (no hypha) through 4 (extensive growth of hypha). The median and the standard deviation were calculated according to standard formula.

Declarations

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In accordance statement concerning research involving plants:

Experimental research on plants has been conducted complying with all relevant institutional, national, and international guidelines and legislation, a. o. the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

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Declaration of generative AI and AI-assisted technologies in the writing process:

During the preparation of this work, the author used Grammarly to improve the English language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Figures

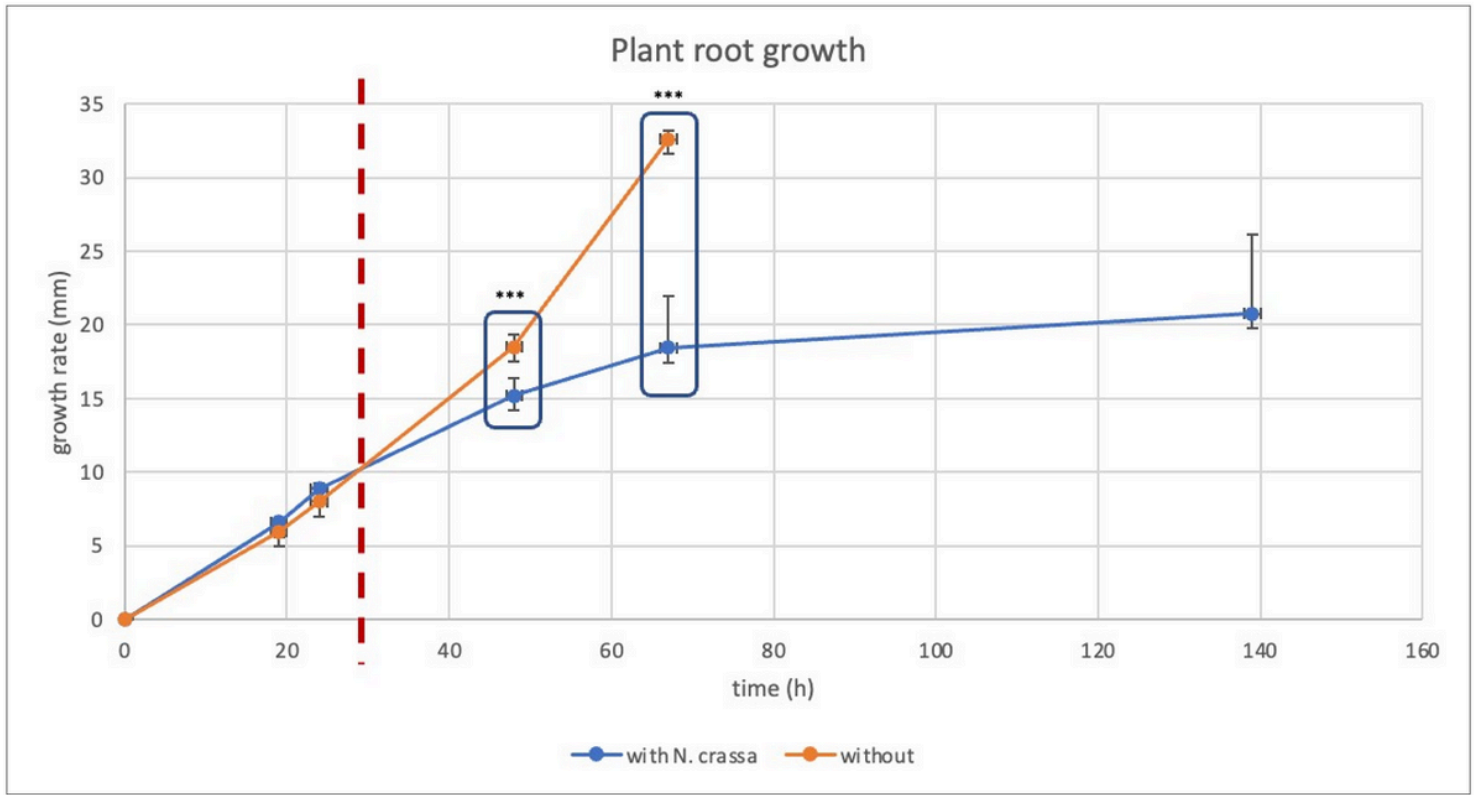


Figure 1

Root growth of *B. distachyon* with (blue) and without (orange) *N. crassa* co-cultivation. The Red dashed line represents the time point, at which *N. crassa* spatially reaches the roots in the co-cultivation experiment. Significant differences between roots in the same developmental stage (grouped by boxes) are depicted as *** ($p < 0.005$)

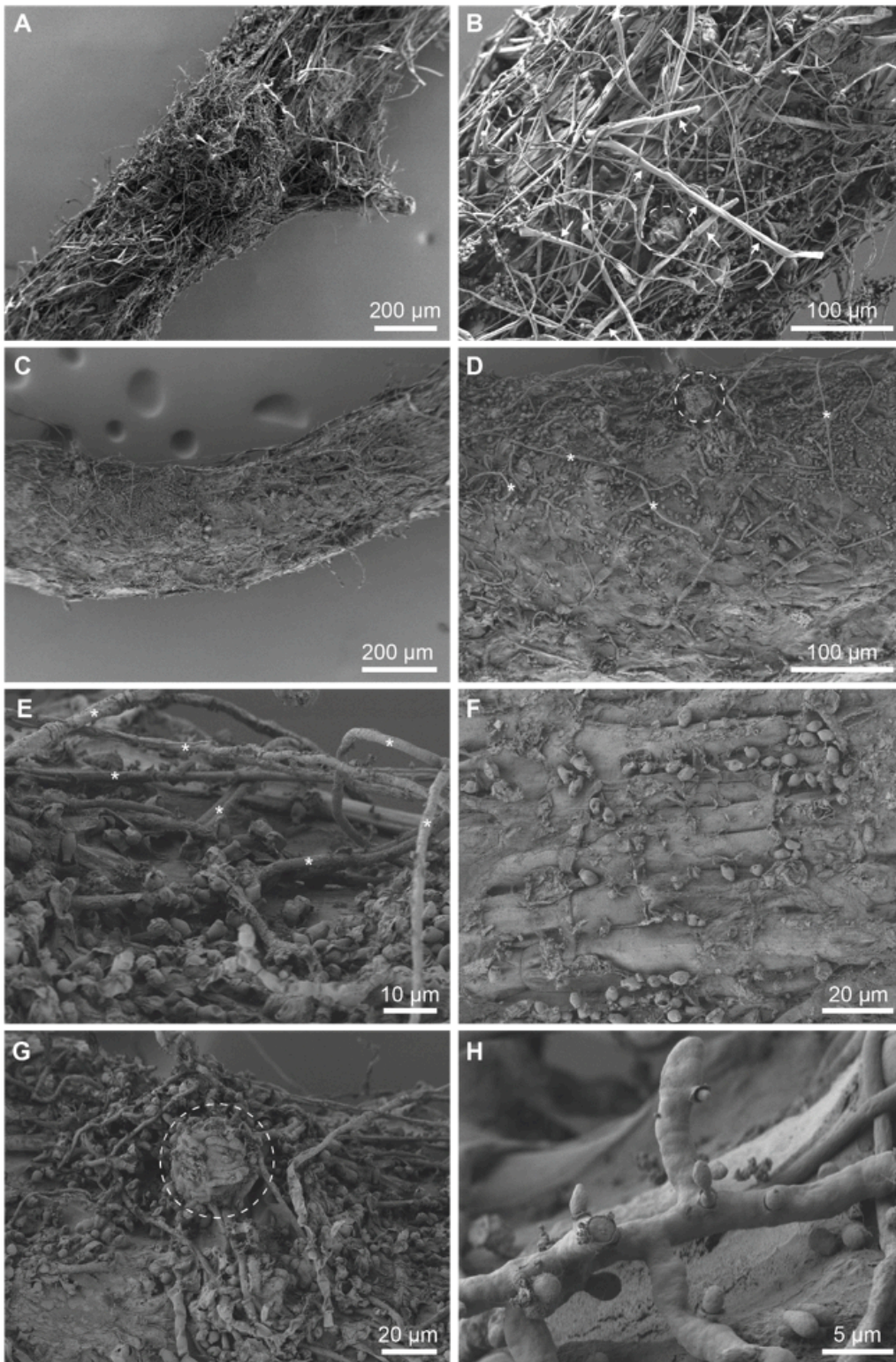


Figure 2

SEM visualization of *B. distachyon* roots infected with *N. crassa*. Roots are wrapped in a dense network of hyphae (A-D) and scattered with oval-shaped conidia (C-H). Hyphae of different thickness can be observed (E). The integrity of the root surface is preserved (F). Protoperithecia (encircled) form in the vicinity of a root (B, D, G). Conidia germlings (H). Asterisk (*): hypha; **circle**: protoperithecium; **white arrow**: root hair.

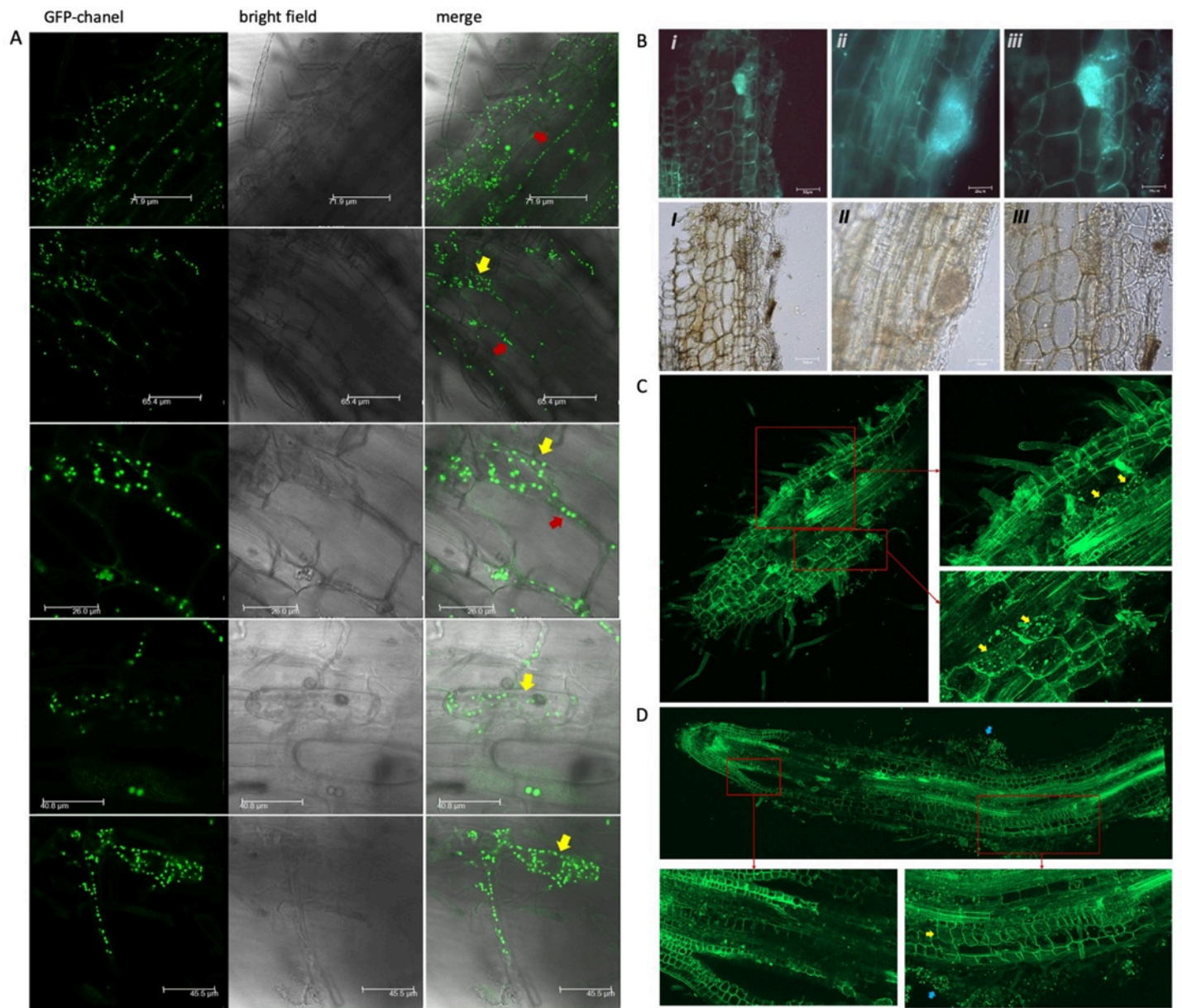


Figure 3

Fluorescent images of longitudinal *B. distachyon* root sections co-cultivated with *N. crassa* FGSC #9518 strain, expressing GFP-coupled histone1 protein. Additional green fluorescence results from auto-fluorescing plant cell wall. **A:** Confocal laser scanning images show apoplastic (**red arrows**) and subcellular (**yellow arrows**) growth of fungal hyphae. **B:** Fluorescent microscopic images show *N. crassa* accumulation in single cortical plant cells. **i-iii.** GFP channel, **I-III.** corresponding brightfield images, **C** and **D:** Spinning-disc confocal microscopy was used to present an overview of a larger root section area. Distinct sectors (depicted by **red boxes**) with invasive *N. crassa* growth (yellow arrows) are magnified. **Blue arrows** indicate the extensive hyphal network covering the root surface.

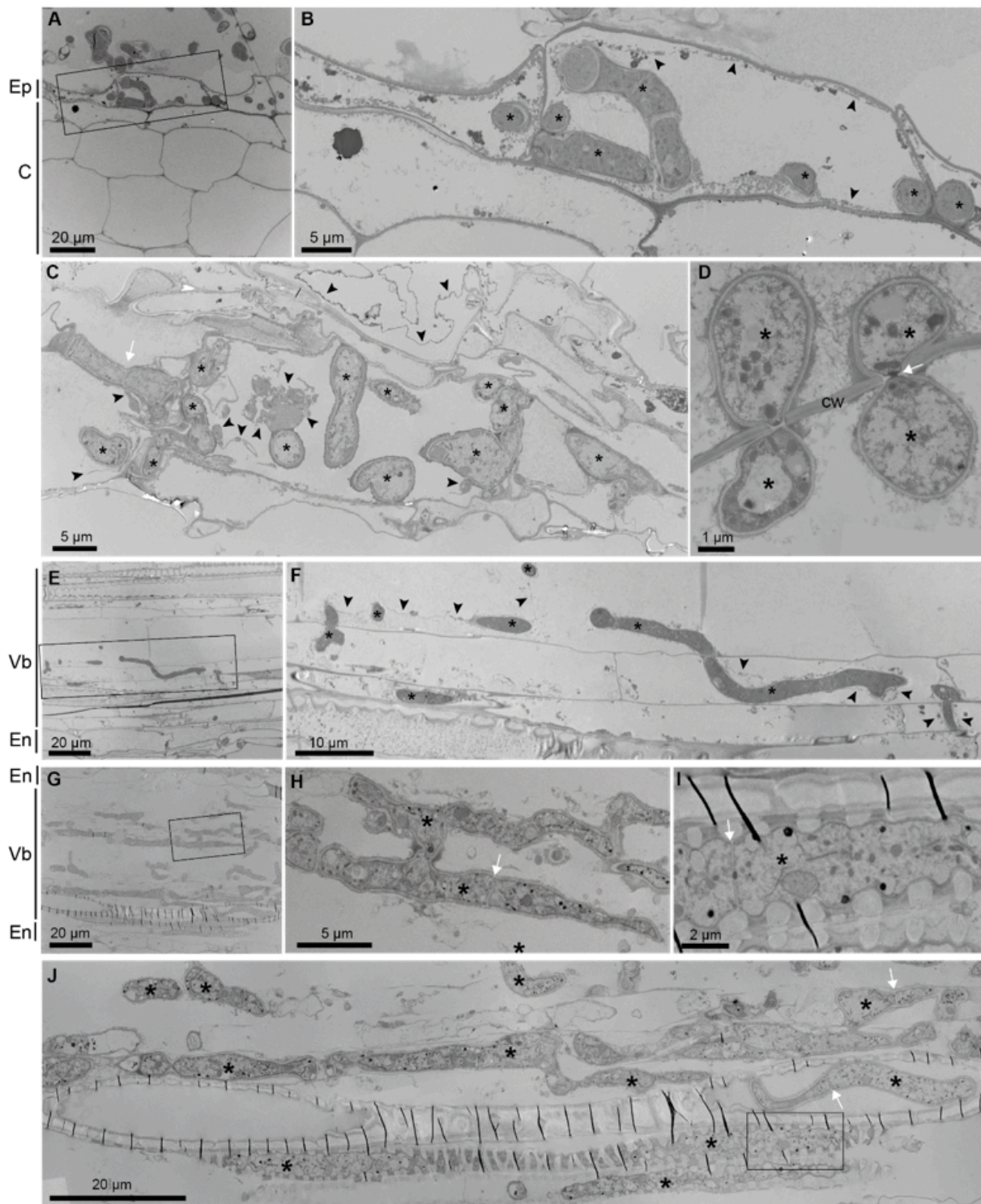


Figure 4

TEM analysis of *B. distachyon* roots infected with *N. crassa*. Hyphae in epidermal cells (**A, B**), in cortical cells (**C, D**), in a moderately infected vascular bundle (**E, F**), and in a strongly infected vascular bundle (**G-J**). Plant cell cytoplasm/membrane (**arrowheads**) can be observed in infected cells and neighboring cells (**C**). Hyphae spread through the root by crossing plant cell walls (**D, F**). In a moderately infected root, plant vascular elements are largely preserved (**E, F**). In a strongly infected root, vascular elements are

destroyed except for the xylem elements with secondary wall thickenings (**G, J**). Parallel, thin, black lines represent folds that were generated over these rigid cell walls during sectioning (**G, J**). Hyphae grow unrestricted (**H**) or inside the xylem vascular elements (**I, J**). The characteristic ultrastructure of fungal hyphae (*) includes the cell wall, septa (with pores) (arrows), and dense cytoplasm. Boxed areas (**A, E, G, J**) are shown enlarged (**B, F, H, I**). Asterisk (*): hypha; **white arrow**: hyphal septum; **black arrowhead**: plant cell cytoplasm/membrane; **Ep**: epidermis; **C**: cortex; **En**: endodermis; **Vb**: vascular bundle **Cw**: cell wall.

Supplementary Files

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