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In vivo observation of conidial germination at the oxic–anoxic interface and infection of submerged reed roots by *Microdochium bolleyi*

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Abstract

The underground plant parts of reed (*Phragmites australis*) growing in anoxic soil of the littoral zone of lakes are provided with oxygen via an aerenchyma. Some of this oxygen is released into the rhizosphere, which creates a potential microhabitat for aerobic fungi. Although fungal endophytes of reed have been shown to occur also in roots of flooded habitats, it is not known whether or how fungi can infect roots growing in anoxic or hypoxic soil. To study fungal infection of reed roots in the laboratory, we developed an incubation chamber to expose reed roots to conidia of *Microdochium bolleyi* in an anoxic agar medium and to observe fungal infection in vivo. Germination rates of conidia were high close to living roots, but decreased to zero in anoxic areas of the chamber. Conidial germ tubes located up to 200 μm from the roots grew preferentially towards the living roots. Conidia also germinated close to air-filled Teflon tubes and exhibited germ-tube tropism, but not as distinctly as on living reed roots. Conidia did not germinate in the neighbourhood of dead roots in anoxic agar. However, in the aerated margin of the incubation chamber most conidia germinated and exhibited tropic growth towards dead roots. Penetration of *M. bolleyi* through several cell layers of living roots was observed in cryo-microtome sections. Penetration was significantly deeper with illuminated plants than with plants kept in the dark; in some cases even the stele was reached. This is the first observation of oxygen released from roots to support growth of an aerobic fungus and of fungal penetration into root tissue in an anoxic environment.

Keywords: *Phragmites australis*; Infection; *Microdochium bolleyi*; Oxic; Anoxic; Rhizosphere

1. Introduction

Reed (*Phragmites australis* (Cav.) Trin. ex Steudel) grows on the shores of Lake Constance (Germany). In this habitat, as in all flooded soils, subterranean plant parts (rhizomes and roots) are surrounded by an anoxic environment [1]. Measurements of oxygen partial pressure in littoral sediments of Lake Constance have revealed that anoxic conditions are reached already within a few millimetres below the sediment surface [2].

Reed plants are well adapted to anoxia. An internal gas transport via aerenchyma provides roots and rhizomes with essential oxygen from shoots and ensures aerobic respiration of the root tissue [3]. It has been shown that the internal oxygen partial pressure of roots placed in anoxic medium ranges from 10 to 12 kPa [4].

Some of the oxygen transported to the lower parts of reed is released into the rhizosphere; this forms an oxygen gradient around the roots [4]. Oxygenation of the root environment occurs most rapidly from young adventitious, secondary and basal tufts of fine lateral roots, but not from older parts of adventitious roots and rhizomes, except for sprouting tips [5].

Oxygen supplied by plants promotes microbial processes in anoxic sediments, such as oxidation of organic substrates or of reduced electron acceptors of anaerobic respirations [6]. It is likely, that this oxygen source also supports growth of aerobic fungi in the rhizosphere and root infection by fungi, as indicated by the rich mycoflora as-

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sociated with submerged reed roots from Lake Constance [7]. Fungal activity was also detected in roots of *Spartina alterniflora* growing in anoxic soil [8]. However, fungal infection processes of roots growing in anoxic environments have not yet been observed or investigated.

One of the fungi frequently isolated from reed roots, especially of flooded habitats on Lake Constance, is *Microdochium bolleyi* (Sprague) de Hoog and Hermanides-Nijhof 1977, Synonym: *Idriella bolleyi* (Sprague)) [7]. *M. bolleyi*, a common soil fungus and coloniser of numerous plants, especially graminaceous species, is generally considered to be nonpathogenic or a weak pathogen [9]. In contrast, the fungus may control take-all disease (*Gaeumannomyces graminis*) [10] and root and even leaf infection by *Bipolaris sorokiniana* [11].

Because of its proven ability to colonise stems and roots of many plants, we used *M. bolleyi* to demonstrate that fungi may use the oxygen released by roots to grow and to infect plants in an anoxic environment.

2. Materials and methods

2.1. Plant and fungal material

Rhizome segments of *Phragmitis australis* with one sprout and newly developed roots (plantlets) were obtained from reed plants grown from surface-sterilised seeds (70% ethanol, 5% NaOCl, 70% ethanol, 5 min each; 3× sterile distilled water) [12] in a green house (18–22°C, daylight). Rooting was carried out in darkened jars with tap water supplemented with streptomycin (100 μg ml^{-1}).

M. bolleyi (strain A7) was isolated from reed roots from Lake Constance and the ITS region of the ribosomal DNA including the 5,8S rRNA gene was sequenced (accession number AJ279454) [7]. For infection experiments, conidial solutions were obtained by rinsing conidia from 10–14-day-old malt extract agar cultures (2% agar, 2% malt, pH 7) with sterile distilled water.

2.2. Germination and infection experiments

An incubation chamber (Fig. 1) was developed that allowed roots and conidia to be studied closely at low oxygen partial pressures. A constant flow of nitrogen (20 ml min^{-1}) through the chamber produced anoxic conditions within the agar.

2.2.1. Plant experiments (12 replicates)

One root of a reed plant was inserted through the opening of the anoxic incubation chamber. The plant base and the rhizome segment were moisturised by means of wet tissue paper. The chamber, whose bottom was closed by a cover slip, was filled with water agar (1% agar, tap water) containing conidia of *M. bolleyi* (3×10^6 conidia ml^{-1}). The medium was bubbled with nitrogen before use. The margin of the chamber was kept free from agar by means of a spacer. The root was positioned close to the lower cover slip by a pair of forceps. After the agar became solid, the spacer was removed, and the agar covered by the upper cover slip. The chambers with the plantlets were cultivated at 20°C ± 1. Sprouts of the reed plantlets were illuminated (120 μE) or kept in the dark.

After 20 h, the percentage of conidial germination along a lateral root (100–120 μm in diameter) and at regular distances from this root, as well as the percentage of germ tubes growing towards the root were determined by visual observation ung an inverted microscope (Zeiss, Jena, Germany) with objectives Plan 10/0.22 and Plan 40/0.65 (Zeiss, Jena, Germany). One single root from each plant situated at the centre of the chamber and 200 spores of each variant were analysed (Fig. 1), data are presented as the mean ± standard deviation.

2.2.2. Dead-root experiments (six replicates)

Autoclaved reed root segments (20 mm long with lateral roots) were placed inside the conidia-containing agar in the anoxic centre of the incubation chamber and in the aerated area 2–4 mm from the open side of the chamber.

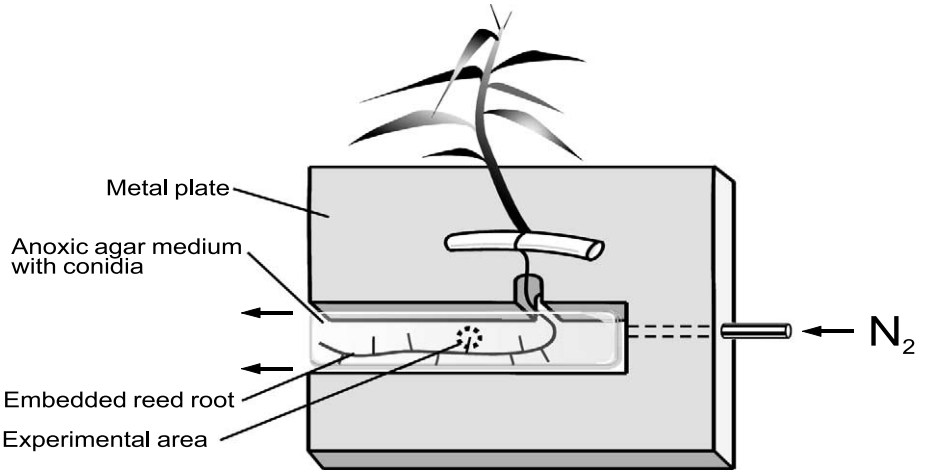

Fig. 1. Scheme of the anoxic incubation chamber (stainless steel, 100×70×2 mm) including a reed plant together with the roots. The agar, surrounding the roots, was flushed with N_2 at the margins and covered with cover slips on both, the upper and the lower side.

2.2.3. Root dummy experiments (six replicates)

In order to imitate an oxygen releasing root, Teflon tubes (inside diameter: 1 mm, sides: 0.25 mm, length: 20 mm) were embedded within the agar with one end exposed to ambient air.

2.3. Cryo-microtome sections and fluorescence microscopy

To observe penetration of the fungus into root tissue, the reed roots embedded in agar were removed from the plant and dissected into smaller pieces, which were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS: 135 mM NaCl, 25 mM KCl, 10 mM Na_2HPO_4, pH 7.3) for at least 30 min. After rinsing in PBS, samples were treated in sucrose solutions (5, 10, 20, and 30% in PBS 30 min each) for cryoprotection.

Specimens were imbedded into Tissue Tec (Sakura, Torrance, CA, USA), frozen on a sample holder at −20°C, and cut into 20-µm sections (Cryostat, CM 1900, Leica, Benzheim, Germany). Sections were mounted on poly-l-lysine coated slides (Sigma, Heidelberg, Germany) and air-dried.

After removing Tissue Tec by washing with PBS (5 min), sections were incubated 45 min with tetramethylrhodamine-isocyanate-labelled wheat germ agglutinin (Sigma, 2% in PBS) to visualise fungal structures. Samples were rinsed two times for 5 min with PBS. Preparations were imbedded in glycerine (50% in PBS).

For microscopical measurement of the depth of penetration of *M. bolleyi* into root tissue, and for determination of the number of penetrated cell layers, an Axioscope fluorescence microscope (Zeiss, Jena, Germany) with a fluorescent filter set (excitation: 546 nm, emission: 570–650 nm) and objectives Plan-Neofluar 10×/0.30, 40×/0.75, and 63×/1.25 oil-immersion (Zeiss, Jena, Germany) were used. For each variant, 10 cross sections from each of six roots were analysed.

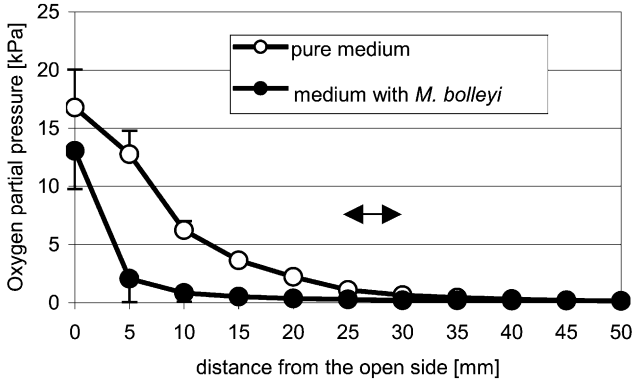

Fig. 2. Oxygen partial pressure in the anoxic incubation chamber filled with agar, in the presence or absence of conidia. The double-headed arrow indicates the position selected for experiments.

2.4. Oxygen measurements

After a constant flow of nitrogen for 20 h, the oxygen partial pressure was measured with oxygen microsensors in agar with and without conidia of *M. bolleyi* in the chamber. Clarke-type oxygen microsensors with guard cathodes [13] had tip diameters of 10–15 µm and 90% response times of <5 s and were constructed and calibrated as previously described [14]. Microsensors were driven into the agar between the two cover slips from the open side at 5-mm increments by using a manual micromanipulator (Narishige, Tokyo, Japan); progress of the tip was followed by means of the microscope. The experiment was carried out in three replications with three parallel tracks per chamber.

3. Results

3.1. Incubation chamber for the observation of fungal development

The cover slips on both sides of the incubation chamber (Fig. 1) allowed the observation of conidia of *M. bolleyi* and roots of *P. australis* with high aperture (N.A. ≤ 0.6) lenses. A constant flow of nitrogen removed the oxygen from the agar surrounding conidia and plant roots.

As a result, the oxygen partial pressure in the agar fell from ~15 kPa to <2 kPa at about 20 mm from the open side of the chamber. After the addition of conidia of *M. bolleyi* to the agar, this value was reached already within 5–10 mm from the open side (Fig. 2).

Before and during the experiment, plants were illuminated or kept in the dark at constant temperature. Thus, we were able to study the impact of oxygen, released by the roots, on the germination of conidia and tropism of germ tubes. Penetration and colonisation of roots was studied after sectioning of such roots and observation of fluorochrome-stained hyphae with a fluorescence microscope.

3.2. Germination of conidia and germ-tube tropism

No germination of conidia was found within the anoxic zone of the agar (Fig. 3a). Only at the aerated outermost margin of the open side, conidial germination was observed. Here, the percentage of germination reached 82% ± 13.

Close to the roots and within the anoxic zone, conidial germination was studied up to a radial distance of 2 mm from the root apex. Highest germination was observed in the immediate vicinity of the root apex of plants kept in the dark. Germination decreased continuously with increasing distances from the root surface and reached values below 2% at a distance of 2 mm (Fig. 3b).

Illumination of the plant leaves (sprouts) had no signifi-

cant influence on conidial germination at the root apex. Again, germination decreased continuously with increasing distances from the root (Fig. 3b).

Percentage of germination along the root surface as well as the decline in germination with increasing distances from the root were similar to that on the root apex (results not shown).

Autoclaved roots did not support any germination of conidia within the anoxic area of the incubation chamber. However, around root segments within the aerated margin of the chamber (open side), 98% ± 3 germination was observed, and germ tubes grew straight towards the roots (97% ± 5).

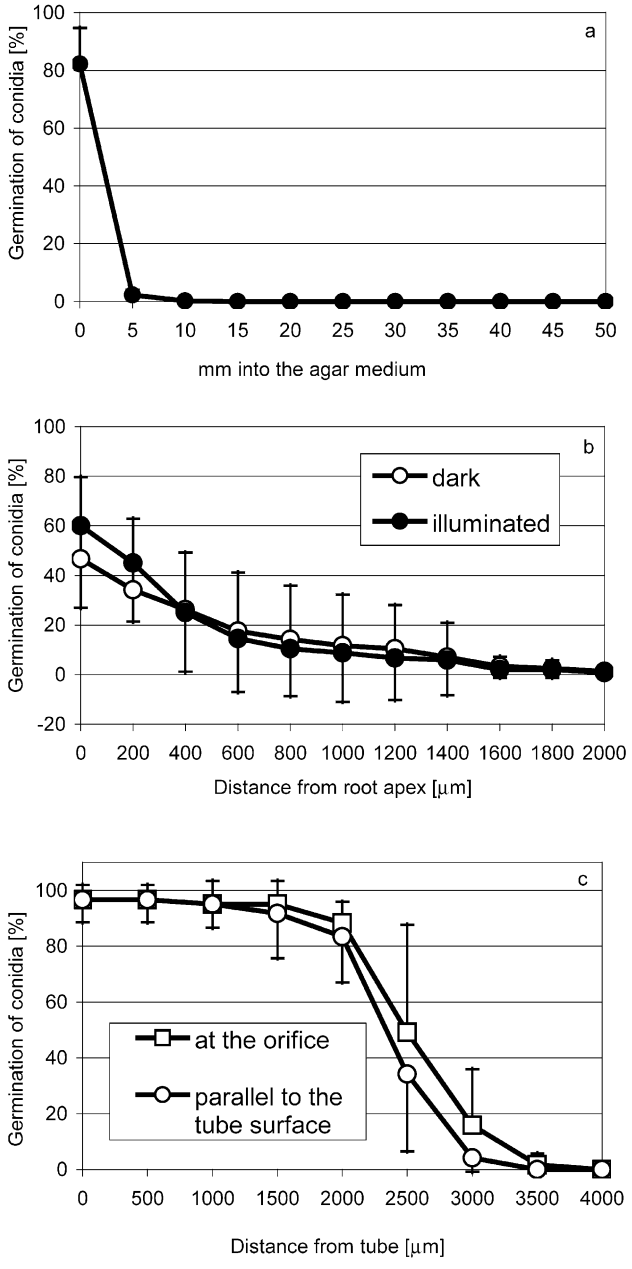

Fig. 3. a–c: Percentage of germination of *M. bolleyi* conidia in the incubation chamber (a) without plants, (b) at different radial distances from the root apex (plant shoots kept in the dark or illuminated), and (c) at increasing distances from the orifice of the Teflon tube or parallel to the tube surface.

Table 1
Number of hyphae that penetrated each cell layer of the reed roots

Cell layer	Number of hyphae that penetrated each cell layer (out of 60)	
	dark	illuminated
Rhizodermis	60	60
Cortex-layer 1	15	28
Cortex-layer 2	1	22
Endodermis	0	14
Stele	0	4

Plant shoots were either kept in the dark or illuminated.

Small Teflon tubes were used as root dummies. Up to a distance of 2 mm from the tube surface and the orifice, respectively, almost all conidia germinated (Fig. 3c). At greater distances, germination rapidly declined.

Germ tubes of conidia grew preferentially towards the living roots or towards the Teflon tubes. With the roots, 88% ± 16 of the germ tubes exhibited tropism towards the root within a distance of 200 µm (Fig. 4a). At greater distances, germ tubes mainly grew undirectionaly. With the Teflon tubes, only 60% ± 12 of the germ tubes grew towards the orifice or the surface. Germ tubes typically exhibited an undulated shape, sometimes like a corkscrew (Fig. 4a,b).

3.3. *Infection structure differentiation and root colonisation*

After growth towards the root (Fig. 4a), appressorium-like structures were produced upon contact with the root surface (Fig. 4b) and seemed to initiate penetration. Twenty hours after root contact, all infection hyphae (out of 60 observed) had colonised the rhizodermis. Hyphal growth proceeded much better in the roots of illuminated plants: four hyphae out of 60 reached the stele within 20 h (Table 1). Immunofluorescence of hyphae in cross sections revealed the whole root was colonised by the fungus (Fig. 4c). We did not observe necrotic cells or tissue. With the plants kept in the dark, only cortical tissue was colonised by hyphae during the period studied (Fig. 4d).

4. Discussion

We have examined conidial germination, including germ tube orientation, in the steep oxygen gradient around reed roots [4], and the penetration of roots by germ tubes of *M. bolleyi* under different light regimens of the shoot. An incubation chamber built for this purpose met the demands for simulating the typically anoxic conditions around submerged reed roots. Measurements with oxygen microsensors confirmed that the continuous flushing with nitrogen gas, supported by the oxygen consumption of the

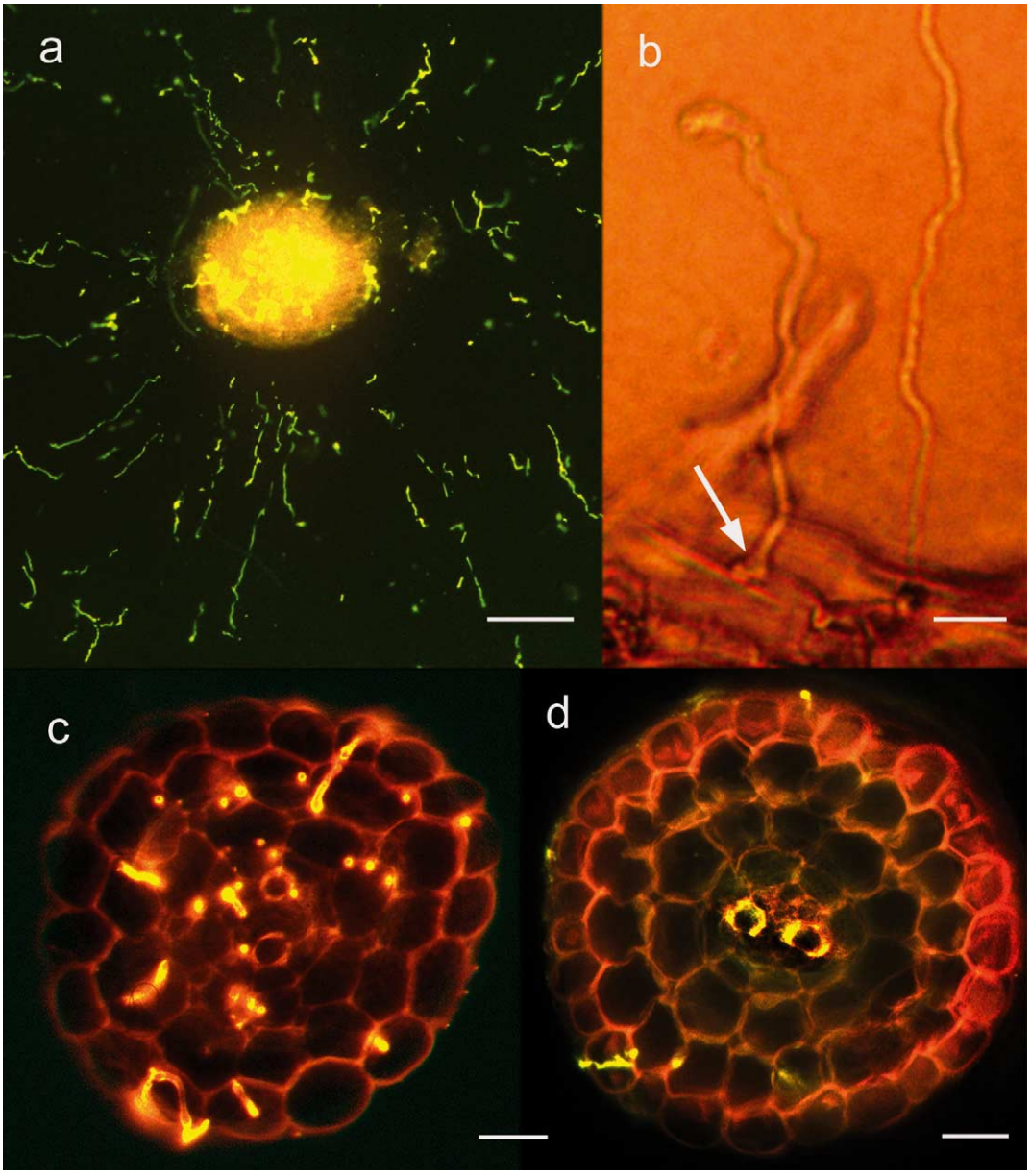

Fig. 4. a: Cross section of a reed root with germ tubes exhibiting tropic growth (fluorescence microscopy, bar = 50 µm), b: germ tube growing towards a reed root and producing an appressorium on the reed surface (bright field microscopy, bar = 20 µm), c: cross section of a reed root from an illuminated plant, profusely colonised by *M. bolleyi* (fluorescence microscopy, bar = 10 µm), and d: cross section of an infected reed root from sprouts, kept in the dark (fluorescence microscopy, bar = 10 µm).

conidia, led to anoxic conditions except at the open end of the chamber exposed to the atmosphere.

Under these conditions, conidia of *M. bolleyi* did not germinate. Lascaris and Deacon [15] have already observed inhibition of conidial germination due to oxygen deficiency by placing a cover slip on conidia embedded in agar. These results and our experiments presented here show that *M. bolleyi* is strictly aerobic. Since *M. bolleyi* is not able to germinate under anoxic conditions, growth of the fungus in the anoxic medium can only be a result of oxygen released by the reed roots. Armstrong et al. [4] measured oxygen partial pressure around reed roots in an anoxic medium. The resulting profiles exhibited an oxygen gradient in the rhizosphere, which coincides with the direction of conidial germination in the present study (Fig. 3).

In addition, *M. bolleyi* exhibited germ-tube tropism towards living reed roots in the anoxic environment. Tropic responses play an important role in pathogenesis by plant pathogenic fungi [16]. Germ-tube tropism of fungi has been described frequently, and attractants for fungal germ tubes may differ. For example, Bordallo et al. [17] have observed a chemotropic response of *Arthrobotrys oligospora* towards wheat, pea, and mustard roots and supposed that substances from the root surface were responsible. Robinson [18] has observed positive chemotropic

responses of germ tubes of *Geotrichum candidum* to oxygen. Marked tropic responses to oxygen occur only when the oxygen partial pressure begins to limit conidial germination and extension-growth of the emergent germ tubes [18]. In our experiments, germ-tube tropism of *M. bolleyi* was restricted to the immediate vicinity of reed roots, which must be a consequence of the marked oxygen gradient. However, root exudates may serve as additional attractants. This was suggested already by studies of Allan et al. [19] who had observed tropic growth of germ tubes of *M. bolleyi* towards dead root hairs of cereals. In order to differentiate between oxygen and further attractants responsible for the tropism of *M. bolleyi*, Teflon tubes and dead reed roots were used to imitate the function of living roots as mere oxygen or nutrient sources, respectively. The abundant germination and the germ-tube tropism towards root dummies, which enable access to oxygen in anoxic environment but does not provide a nutrient source, point also at oxygen as one important attractant for *M. bolleyi*. In contrast, dead reed roots, which were used to test roots as a nutrient source, did not enable germination of conidia under anoxic conditions. This result was not surprising because of the oxygen requirements of the fungus. However, the strong tropic behaviour of germ tubes on dead roots in oxic zones of the medium suggest that root exudates of *P. australis* provide additional attractants for germ tubes of *M. bolleyi*.

Germ-tube tropism and the differentiation of appressoria prepared the penetration into the roots. Cross sections revealed fungal structures in the root and confirmed the assumption that *M. bolleyi* is a benign endophyte [7,20,21]. In most cases, the fungus penetrated only the first epidermal layer. However, hyphae reached the stele after illumination of the shoot. Hodges and Campbell [22] have observed hyphal growth of *M. bolleyi* in the cortex of inoculated adventitious roots of *Agrostis palustris*, but vascular tissue was not penetrated. Because our experiments were terminated after 20 h and the individual experiments exhibited a highly variable percentage of germination, we assume that penetration up to the stele is generally possible, and that the penetration depth depends on the speed of germination and penetration of the fungus.

Observed differences are probably due to varying oxygen emission of the reed roots. In illuminated samples, germination of *M. bolleyi* conidia on the roots was slightly elevated and fungi penetrated deeper into the root tissue. Armstrong and Armstrong [23] have detected an enhancement of rhizome and root aeration owing to an increasing illumination, which enhances oxygen partial pressure in the aerenchyma and release of oxygen into the rhizosphere. This increased oxygen supply is believed to be the reason for an accelerated penetration of root tissue in illuminated plants.

The main results of this study can be summarised as follows:

- By using the newly designed anoxic incubation chamber, we present evidence that oxygen is the most important factor for fungal infection of roots in anoxic soil. Illumination of the plant enhances this effect.
- Endophytic fungi can colonise roots under anoxic conditions by using oxygen from the plant, as shown here for *M. bolleyi*. Germ-tube tropism seems to be important for finding the host in this environment. The fungus penetrates root cells in anoxic soil not only to acquire assimilated compounds for heterotrophic nutrition, but also to acquire oxygen for maintaining aerobic respiration. In an anoxic environment, an aerobic fungus like *M. bolleyi* is not able to live saprophytically. It can only live as a biotroph inside a living root.

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References

[1] Brune, A., Frenzel, P. and Cypionka, H. (2000) Life at the oxic-anoxic interface: microbial activities and adaptions. FEMS Microbiol. Rev. 24, 691–710.
[2] Frenzel, P. (1990) The influence of chironomid larvae on sediment oxygen microprofiles. Arch. Hydrobiol. 119, 427–437.
[3] Jackson, M.B. and Armstrong, W. (1999) Formation of aerenchyma and the processes of plant ventilation in relation to soil flooding and submergence. Plant Biol. 1, 274–287.
[4] Armstrong, W., Cousins, D., Armstrong, J., Turner, D.W. and Beckett, P.M. (2000) Oxygen distribution in wetland plant roots and permeability barriers to gas-exchange with the rhizosphere: a microelectrode and modelling study with *Phragmites australis*. Ann. Bot. 86, 687–703.
[5] Armstrong, J. and Armstrong, W. (1988) *Phragmites australis* - a preliminary study of soil-oxidizing sites and internal gas transport pathways. New Phytol. 108, 373–382.
[6] Liesack, W., Schnell, S. and Revsbech, N.P. (2000) Microbiology of flooded rice paddies. FEMS Microbiol. Rev. 24, 625–645.
[7] Wirsel, S.G.R., Leibinger, L., Ernst, M. and Mendgen, K. (2001) Genetic diversity of fungi closely associated with common reed (*Phragmites australis*). New Phytol. 149, 589–598.
[8] Padgett, D.E. and Celio, D.A. (1990) A newly discovered role for aerobic fungi in anaerobic salt marsh soils. Mycologia 82, 791–794.
[9] Domsch, K.H., Gams, W. and Anderson, T.-H. (1980) Compendium of Soil Fungi. Vol. 1. Academic Press, London.
[10] Dawson, W.A.J.M. and Bateman, G.L. (2000) Sensitivity of fungi from cereal roots to fluquinconazole and their suppressiveness towards take-all on plants with or without fluquinconazole seed treatment in a controlled environment. Plant Pathol. 49, 477–486.
[11] Liljeroth, E. and Bryngelsson, T. (2002) Seed treatment of barley with *Idriella bolleyi* causes systemically enhanced defence against root and leaf infection by *Bipolaris sorokiniana*. Biocontrol Sci. Technol. 12, 235–249.
[12] Ernst, M., Mendgen, K.W. and Wirsel, S.G.R. (2003) Endophytic fungal mutualists: Seed-borne Stagonospora spp. enhanced reed biomass production. Mol. Plant-Microbe Interact., 16, 580–587.
[13] Revsbech, N.P. (1989) An oxygen microsensor with guard cathode. Limnol. Oceanogr. 34, 474–478.

[14] Brune, A., Emerson, D. and Breznak, J.A. (1995) The termite gut microflora as an oxygen sink: microelectrode determination of oxygen and pH gradients in guts of lower and higher termites. Appl. Environ. Microbiol. 61, 2681–2687.
[15] Lascaris, D. and Deacon, J.W. (1994) In vitro growth and microcycle conidiation of *Idriella bolleyi*, a biocontrol agent of cereal pathogens. Mycol. Res. 98, 1200–1206.
[16] Wynn, W.K. (1981) Tropic and taxic responses of pathogens to plants. Annu. Rev. Phytopathol. 19, 237–255.
[17] Bordallo, J.J., Lopez-Llorca, L.V., Jansson, H.-B., Salinas, J., Persmark, L. and Asensio, L. (2002) Colonization of plant roots by egg-parasitic and nematode-trapping fungi. New Phytol. 154, 491–499.
[18] Robinson, P.M. (1973) Oxygen-positive chemotropic factor for fungi? New Phytol. 72, 1349–1356.
[19] Allan, R.H., Thorpe, C.J. and Deacon, J.W. (1992) Differential tropism to living and dead cereal root hairs by the biocontrol fungus *Idriella bolleyi*. Physiol. Mol. Plant Pathol. 41, 217–226.
[20] Hemens, E., Steiner, U. and Schönbeck, F. (1992) Infektionsstrukturen von *Microdochium bolleyi* an Wurzeln und Koleoptilen von Gerste. J. Phytopathol. 136, 57–66.
[21] Reinecke, P. (1978) *Microdochium bolleyi* at the stem base of cereals. Z. Pfl. Krankh. Pfl. Schutz. 85, 679–685.
[22] Hodges, C.F. and Campbell, D.A. (1996) Infection of adventitious roots of *Agrostis palustris* by *Idriella bolleyi*. J. Phytopathol. 144, 265–271.
[23] Armstrong, J. and Armstrong, W. (1990) Light-enhanced convective throughflow increases oxygenation in rhizomes and rhizosphere of *Phragmites australis* (Cav.) trin ex. Steud. New Phytol. 114, 121–128.