Journal für Kulturpflanzen, 69 (9). S. 291–302, 2017, ISSN 1867-0911, DOI: 10.1399/JfK.2017.09.02 Verlag Eugen Ulmer KG, Stuttgart

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Originalarbeit

Detection and growth of endophytic entomopathogenic fungi in dicot crop plants

Nachweis und Wachstum von endophytischen, entomopathogenen Pilzen in dikotylen Kulturpflanzen

Abstract

The presence and distribution of fungal endophytes in plants is commonly assessed by re-isolation on agar media or detection by PCR-techniques. Histological studies on the process of colonization of the host plant have only scarcely been performed. In the present study, the development of entomopathogenic fungi on the plant surface and inside the tissue was examined by light and fluorescence microscopy of leaf samples treated with various dyes or, to guarantee the specificity of injected endophytes, with primary polyclonal and secondary FITC-conjugated antibodies; diaminobenzidine-tetrahydrochloride (DAB) was applied as stress test for the detection of hydrogen peroxide. Four species of entomopathogenic fungi were studied and compared with three phytopathogenic fungal species. The host plants were oilseed rape (*Brassica napus*), faba bean (*Vicia faba*), and cucumber (*Cucumis sativus*).

When blastospores of selected four fungal species were infiltrated into *B. napus* leaves they appeared to germinate only on the leaf surface, but not within the mesophyll. Successful re-isolation from *B. napus* inoculated with *B. bassiana*, *Isaria fumosorosea* or *Metarhizium anisopliae* showed that these entomopathogens were able to persist in the tissue for at least two weeks. Formation of brown precipitates after leaf treatment with DAB in the presence of *B. bassiana* indicated the production of hydrogen peroxide by *B. napus* but not by *V. faba*. Overall, the results indicate a lower endophytic colonization than could have been expected from the literature, suggesting nutrient availability in the plant intercellular space and absence of cell wall and cell membrane degrading fungal enzymes as fungal growth-limiting factors. It is concluded that data on endophytic colonization should generally be supported by histological evidence of the kind and amount of fungal growth in the host tissue.

Key words: Endophytes, *Brassica napus*, *Vicia faba*, entomopathogenic fungi, phytopathogenic fungi, immunofluorescence microscopy

Zusammenfassung

Der Nachweis und die Verbreitung pilzlicher Endophyten in Pflanzen wird üblicherweise durch Re-Isolierung auf Agarmedien oder durch PCR-Techniken erbracht. Histologische Untersuchungen zur Kolonisierung der Wirtspflanze liegen dagegen nur selten vor. In der vorliegenden Arbeit wurde die Entwicklung von entomopathogenen Pilzen auf der Pflanzenoberfläche und innerhalb des Gewebes durch Licht- und Fluoreszenzmikroskopie von mit verschiedenen Farbstoffen behandelten Blattproben untersucht oder, um die Spezifität der injizierten Endophyten zu gewährleisten, mit primären polyklonalen und sekundären FITC-konjugierten Antikörpern. Diaminobenzidin-Tetrahydrochlorid (DAB) diente dem Nachweis von Wasserstoffperoxid als Stresstest.

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Accepted
02 August 2017

Vier entomopathogene Pilz-Gattungen wurden mit drei phytopathogenen Pilzarten verglichen. Wirtspflanzen waren Raps (*Brassica napus*), Ackerbohnen (*Vicia faba*) und Gurken (*Cucumis sativus*).

Auch wenn Blastosporen von *Beauveria bassiana* in *B. napus* Blätter infiltriert wurden, schienen sie trotzdem nur auf der Blattoberfläche zu keimen. Die erfolgreiche Pilz-Re-Isolierung aus Blättern von *B. napus*, die mit *B. bassiana, Isaria fumosorosea* oder *Metarhizium anisopliae* inokuliert wurden, bewies ein Überdauern dieser Entomopathogene im Gewebe für mindestens zwei Wochen. Bildung brauner Niederschläge nach der Blattbehandlung mit DAB in Gegenwart von *B. bassiana* zeigte die Produktion von Wasserstoffperoxid in *B. napus* an, aber nicht in *V. faba*. Insgesamt zeigen diese Ergebnisse eine niedrigere endophytische Kolonisierung, als nach der Literatur zu erwarten, was auf eine geringe Verfügbarkeit von Nährstoffen im pflanzlichen Interzellularraum und die Abwesenheit von Zellwand- und Zellmembran-abbauenden pilzlichen Enzymen als wachstumsbegrenzende Faktoren hindeutet. Daten über die endophytische Kolonisierung sollten daher allgemein jeweils durch histologische Nachweise von Art und Ausmaß des Pilzwachstums im Wirtsgewebe unterstützt werden.

Stichwörter: Endophyten, *Brassica napus*, *Vicia faba*, entomopathogene Pilze, phytopathogene Pilze, Immunfluoreszenz-Mikroskopie

Introduction

Due to problems with resistance development of insect pests against chemical insecticides (Bass et al., 2014; Sparks and Nauen, 2015; Buzetti et al., 2016), the ban of certain groups of pesticides (Hillocks, 2012; Stockstad, 2013) and the demand of the public for non-chemical plant protection methods, there is an increasing interest in the use of entomopathogenic fungi in biological plant protection. A number of biocontrol preparations based on entomopathogenic fungi are commercially available. They commonly contain high numbers of fungal spores and are applied onto plant surfaces by spraying (Zimmermann, 2007; Oliveira et al., 2015). A number of reports in the literature describe the isolation of *Beauveria bassiana*, *Metarhizium anisopliae* and other entomopathogenic fungi from plants, suggesting an endophytic life style of these fungi (Vega et al., 2008; Ownley et al., 2010). Infection of insect pests via plant tissues colonized by endophytes, ideally combined with seed transmission of the latter, would be an elegant way of delivery. Therefore, attempts are being made to experimentally introduce entomopathogenic fungi into crop plants. Probably the best studied example in this respect is the entomopathogen *B. bassiana*. Its establishment as endophyte has been reported for many plant species (Jaber, 2015; Vidal and Jaber, 2015; Greenfield et al., 2016). It can be expected that to be effective against herbivores, long term establishment of the endophyte in the plant at sufficient densities is important. Re-isolation on agar media and detection by PCR-techniques are at present the preferred methods for monitoring the presence and distribution of endophytes in plants. It has been pointed out that the study of endophytes is very much a method-dependent process (Hyde and Soytong, 2008).

Histological observation (e.g. Wagner and Lewis, 2000; Gómez-Vidal et al., 2006; Landa et al., 2013) appears to be only rarely performed (McKinnon et al., 2017), despite the fact that it is the best method to evaluate to what extent a fungus actually colonizes the host (Schulz and Boyle, 2005). It not only provides information on the spatial relationship between host and endophyte but may also elucidate physiological reactions associated with host defence.

In order to contribute to a better understanding of the relationship between endophytic entomopathogens and potential hosts, a study was initiated that aimed at documenting the development of different entomopathogenic fungi in the important dicot crop plants *Brassica napus*, *Vicia faba* and *Cucumis sativus*. Following inoculation of plants with blastospores or conidiospores the development of the fungi was studied on the plant surface and inside the tissue by light and fluorescence microscopy. The fungal structures were visualized using common dyes as well as polyclonal antibodies labelled with a fluorescence marker. The work was complemented by re-isolation assays that monitored the survival of the endophytic fungi in the plant tissue.

Materials and Methods

Plant material

Experimental plants were *Brassica napus* L. cultivars Aviso (Lantmännen SW-Seed, Teendorf, Germany), Licolly (Deutsche Saatveredelung, Lippstadt, Germany), Adriana (Limagrain, Edemissen, Germany), Laser, Fortis and NK Jetix (Syngenta Seeds, Bad Salzuflen, Germany), *Cucumis sativus* L. cv. Chinesische Schlange (ENZA, Zaden, The Netherlands) and *Vicia faba* L. cv. Espresso (H.G.-Lembke, Malchow/Poel, Germany). The plants were grown in standard potting soil (Fruhstorfer Erde Typ LD 80, HAWITA Gruppe GmbH, Vechta, Germany) for up to 3 months either at 15 – 25°C in a greenhouse or at 20°C in a growth cabinet under fluorescent tubes or metal halide lamps (16/8 h, 150 – 240 µmol sec^{-1} m^{-2}). Sterile *V. faba* seedlings were raised in magenta boxes (Sigma) on malt peptone agar (MPA) (30 g malt extract, 5 g soybean peptone, 18 g agar per 1000 ml dH_2O).

Entomopathogenic fungal strains and inoculation of plants

All entomopathogenic fungal strains were obtained from the culture collection of the Institute for Biological Control (Darmstadt). *Beauveria bassiana* strains ATTC 74040 (re-isolate of the product NATURALIS®), JKI-BI-1202 and JKI-BI-1133 were grown on MPA. *B. bassiana* NATU-

RALIS was reported to be a successful endophyte (Vidal and Jaber, 2015). Blastospores of *B. bassiana* were produced in 300 ml flasks in 50 ml Czapek-Dox broth (Sigma-Aldrich) (Czapek, 1902; Dox, 1910). After cultivation on a rotary shaker for 3 days at 25 °C the cultures were filtered through gauze (Mullro®) and the concentration of blastospores was adjusted to 1×10^5–1×10^8/ml, either after pelleting of the spores by centrifugation or by directly diluting the cultures with sterile tap water. After confirming the spore viability with 0.01% acridine orange under blue fluorescent light (Strugger, 1940; Darzynkiewicz et al., 1975; Matecki et al., 2015), the suspensions of blastospores were used for inoculation of true leaves of *B. napus*, *V. faba* or *C. sativus* by tissue infiltration (Klement, 1990) or by injection into stems (Matecki et al., 2015). Tissue infiltration was achieved by gently pressing the open end of a 5 ml syringe (without hypodermic needle) on the lower side of the leaf and applying sufficient pressure to introduce the blastospore suspensions through the stomates into the leaf. Additionally, faba bean seeds were surface sterilized by 1% sodium hypochlorite (10 min) and 70% ethanol (2 min). Seeds were washed 3 times with sterile tap water (2 min each). Seeds were coated with spore suspensions in a shaking Intelli-Mixer (Neolab GmbH) for four days at 6 rpm and 25 ± 2 °C (16/8 h, RF 58%). Then the seeds were transferred into sterile magenta boxes containing MPA. The entomopathogenic fungal species *Isaria fumosorosea* JKI-BI-1496, *Isaria farinosa* JKI-BI-1495, *Lecanicillium muscarium* JKI-BI-1553 and *Metarhizium anisopliae* JKI-BI-1339 were cultured in Samšináková (1966) medium (25 g glucose, 20 g corn steep solid, 5 g NaCl per 1000 ml dH_2O) and similarly applied as *B. bassiana*.

Fungal staining and microscopy

From inoculated leaves cross and paradermal hand-cut sections were prepared and cleared with chloral hydrate solution (1.0 g/ml dH_2O)/90% lactic acid (2:1) for one to three days. After thoroughly rinsing with dH_2O, the sections were stained with 0.01% blankophor (Bayer, Leverkusen, Germany) in 0.1 M Tris buffer pH 9.0 for 3 min or with 0.1% solophenyl flavine 7GFE (syn. Direct yellow 96; Shijazhuang Kun Li chemical, China) in 0.1 M Tris/HCl pH 8.5. Prior to staining with the latter dye, the sections were pre-stained with 0.05% safranin for 5 min and rinsed with buffer in order to diminish tissue fluorescence (Knight and Sutherland, 2011). Samples were examined under UV (Leica-filter block A, excitation 340–380 nm, emission ≤ 430 nm) with an Aristoplan epifluorescence microscope (Leica, Wetzlar, Germany). Images were digitally taken with a CCD camera (ColorView II, Olympus) using the software AnalySIS FIVE. The third method used was staining with trypan blue. Leaf segments were boiled in the staining solution (10 ml lactic acid, 10 ml glycerol, 10 ml water, 10 g phenol, 10 mg trypan blue, Merck) for two to four min. After clearing in 100% chloral hydrate solution for at least 30 min, the samples were mounted in glycerol, and microscopically viewed under differential interference contrast optics (Koch and Slusarenko, 1990).

Immunolocalization of fungi

The above described staining techniques are useful to detect fungi in general. To guarantee specific detection of exclusively *Beauveria* or *Isaria* and no other fungi, polyclonal antisera against *B. bassiana* (ATTC 74040) and *I. fumosorosea* (JKI-BI-1496) were raised in rabbits. For this purpose, fungal biomass from shake cultures was homogenized, centrifuged, and the supernatant used for immunization. IgG fractions were purified as described previously (Eibel et al., 2005). In Western blotting experiments the main reactive bands for *B. bassiana* and *I. fumosorosea* antigen preparations revealed an apparent molecular weight of approximately 27 or 30 kDa, respectively (data not shown). Leaf tissue sections were fixed for 4 h with 4% (w/v) paraformaldehyde in 0.1 × phosphate-buffered saline (0.1 × PBS: 13.7 mM NaCl, 0.15 mM KH_2PO_4, 0.65 mM Na_2HPO_4, and 0.27 mM KCl, pH 7), washed with microtubuli stabilizing buffer (MTSB: 50 mM HEPES, 5 mM $MgSO_4 \times 7\ H_2O$, 5 mM EGTA, pH 6.9) and 1 × PBS. After dehydration with a graded series of ethanol at 22 °C, tissues were embedded overnight in Steedman's wax, a polyester with a low melting point (PEG 400 distearate and cetylalcohol 9:1 [w/w]), according to the method of Vitha et al. (1997). Cross sections of 10 µm thickness were prepared with a microtome at room temperature (Cryocut CM 3050, Leica, Germany) and collected on albumin-glycerol coated slides. Steedman's wax was removed from sections with decreasing ethanol concentrations, rinsed with 0.1 PBS, incubated with buffer for 30 min, and with 100% methanol for 10 min at −20 °C. For blocking unspecific binding sites, the sections were incubated with 1% BSA (Aurion) in 0.1x PBS for 1.5 h at 22 °C. For immunolabeling, sections were incubated overnight with the anti-*B. bassiana* (fractions 142/4 or 143/4: 1 mg/ml) or anti *I. fumosorosea* IgG (fractions 144/4: 1 mg/ml or 144/4–2/2: 2 mg/ml), diluted 1:200 in 1% (w/v) BSA solution. For fungal labelling, green fluorescent FITC conjugate (goat anti-rabbit IgG, H + L, 2 mg/ml, Molecular Probes, Göttingen, Germany) was used as secondary antibody, diluted 1:200 with 0.1 PBS and applied for 2 h at 22 °C. After immunolabeling, the sections were mounted on slides in 2.5% 1.4-diazabicyclo[2.2.2]octane (DABCO) in glycerol. Control sections were incubated without primary antibodies either with 1% (w/v) BSA or with 1:200 diluted rabbit preimmune serum (# 170) and did not show secondary antibody-caused fluorescence. Sections were examined under fluorescent blue light (Leica-filter block I3, excitation 450–490 nm, emission ≤ 515 nm) with the same microscope and camera system as stated above. Further details were described by Wächter et al. (2003). Altogether, up to 5000 sections (stained with dyes or immunologically labelled) were microscopically analyzed.

DAB oxidative stress test

For examining possible plant defence reactions towards the endophytic fungi, the production of H_2O_2 was investigated following the protocol of Thordal-Christensen et al. (1997). Sections of leaves were taken 4 h to 3 d post

inoculation (hpi, dpi), incubated in 10 mM MES-buffer, pH 6.0 for 2 h and then overnight in 0.1% 3.3-diaminobenzidine-tetrahydrochloride (DAB) in darkness. After removing the DAB-solution, leaf sections were incubated in 100% boiling ethanol for 10 min for chlorophyll extraction, followed by overnight incubation in ethanol at room temperature in darkness. The samples were mounted on slides in 70% glycerol and viewed by transmitted-light bright-field microscopy. About 200 sections were analyzed.

Re-isolation experiments

The fungi used were the entomopathogens *B. bassiana* ATTC 74040, *M. anisopliae* JKI-BI-1339 and *I. fumosorosea* JKI-BI-1496, the oil seed rape pathogens *Plenodomus lingam* (teleomorph *Leptosphaeria maculans*) strain T12aD34 and *Plenodomus biglobosus* (teleomorph *Leptosphaeria biglobosa*) strain NA22 (formerly regarded as aggressive and non-aggressive variants, respectively, of the blackleg pathogen *Phoma lingam*) (Shoemaker and Brun, 2001) and a strain of *Ascochyta fabae*. The fungi were cultured on potato dextrose agar (PDA) (*B. bassiana, M. anisopliae, I. fumosorosea, A. fabae*) or V8 medium (*L. maculans, P. biglobosus*). Conidial suspensions (10^7 conidia per ml) were prepared by addition of a few ml of sterile 0.0125% Tween 20 to sporulating plates. Cotyledons of 10-day-old *B. napus cv*. Adriana plants were punctured with a needle (one wound per cotyledon half) and inoculated with 5 µl drops of suspension on each wound, or mock-inoculated with drops of 0.0125% Tween 20. The plants were then returned to the growth room. Cotyledons were harvested 14 dpi and surface sterilized for 2 min each in 0.5% sodium hypochlorite and 70% ethanol, followed by three rinses in sterile dH_2O. Water of the last rinse was plated on PDA to check the sterilization effect. Discs of leaf were excised using cork borers with 6, 8 or 10 mm diameter, respectively and placed on PDA with 0.002% Rifampicin + 0.005% Streptomycin (PDA + Antibiotics). The plates were incubated at 23 ± 2°C and regularly inspected for mycelium growing from the tissue. Alternatively, cotyledons were infiltrated with conidial suspension as described above.

Similarly, the first and the second pinnate leaves of 10-days-old *V. faba* plants were wound-inoculated (two wounds per leaflet) or infiltrated with conidial suspensions of the entomopathogenic fungi or *A. fabae*. Inoculated plants were returned to the growth room and arranged in a randomized design. In order to delay senescence, fresh shoot growth was removed. Fourteen days after inoculation, samples were taken and processed as explained above for oilseed rape cotyledons.

Results

Detection of fungi by staining with blankophor, solophenyl flavine 7 GFE, and trypan blue

All tested dyes effectively stained the examined entomopathogenic fungi, *i.e.* strains of *B. bassiana, I fumosorosea, L. muscarium* and *M. anisopliae* (Fig. 1a-g). Blankophor efficiently bound to the hyphae of all fungi on the leaf surface of *B. napus* (Fig. 1a). On plant surfaces, hyphae were detected up to four weeks after application of spores. Solophenyl flavine 7 GFE effectively stained *B. bassiana* spores and hyphae. It yielded the best contrast after pre-staining the samples with safranin, which suppresses plant cell wall fluorescence (Fig. 1b, c, f). Comparable patterns were obtained with *M. anisopliae* on *B. napus* leaves (Fig. 1e). Trypan blue also provided a good contrast between spores and hyphae on *B. napus* leaf surfaces (Fig. 1g). Fig. 1g shows that spores and hyphae of *B. bassiana* (arrows) were considerably smaller than the width of the stomatal opening.

After infiltration of spores into *B. napus* leaves, blankophor stained hyphae outside the stomates (Fig. 1h, arrow). In cross sections no hyphae were detected inside the tissue. Safranin/Solophenyl 7GFE-staining of the tissue allowed a view into the spongy parenchyma, as shown for uninoculated control tissue of *V. faba* (Fig. 1i). Different from *B. napus*, with this method spores and short hyphae could be detected in the mesophyll of *V. faba* 2 dpi (Fig. 1j, arrowheads).

Detection of fungi by immunolocalization

The fungi examined, *B. bassiana, I. fumosorosea, L. muscarium* and *M. anisopliae,* were specifically detected by the anti-*Beauveria* as well as the anti-*Isaria* IgGs (Fig. 2a-d). Infiltration of the spore suspension from the lower leaf surface into the leaves of *B. napus* was successful and could be followed visibly. Nevertheless, *B. bassiana* hyphae were detected only on the lower leaf surface at the site of infiltration (Fig. 2e-g). Spongy and palisade parenchyma were clearly devoid of hyphae in *B. napus* (Fig. 2f, g, i), as well as the vascular bundles (Fig. 2h).

B. bassiana, I. fumosorosea and *L. muscarium* were infiltrated also into the leaves of *V. faba* and *C. sativus*. In contrast to *B. napus,* the spores of *B. bassiana* germinated in the leaf tissue of faba bean and cucumber, and hyphae were detected growing within the intercellular space along the cell walls (Fig. 2j-l, 3a, b), except within the vascular bundles, where there are no intercellular spaces (Fig. 2h, j). Hyphae also grew in the mesophyll of *C. sativus* after inoculation with *B. bassiana* (Fig. 3c, d). Not only *B. bassiana* but also *I. fumosorosea* was detected within the mesophyll of *V. faba* leaves (Fig. 3e, f).

In samples taken over a period of 4 to 8 weeks after inoculation, less and less fungal hyphae remained detectable on and within bean leaves. Most hyphae had a corroded and starved appearance. Hyphae were neither detected in the main stem of the shoot, nor in the shoot apical meristem (SAM).

DAB staining

To detect H_2O_2 production triggered by fungal hyphae, segments from inoculated leaves were incubated in diaminobenzidine (DAB). By 4 hpi, brownish hyphae within *B. napus* stomates indicated the plant production of H_2O_2 upon contact with *B. bassiana* hyphae,

resulting in the brown precipitation due to dimerization of DAB (Fig. 4a). The reaction was even stronger 1 dpi (Fig. 4b–d, arrows). In the non-inoculated control leaves, hence in the absence of fungal hyphae, no brown coloured guard cells of the stomates were detected (Fig. 4e). The reaction of *B. napus* towards *I. fumosorosea* and *L. muscarium* was similar as towards *B. bassiana*. The response was still detectable 7 dpi (Fig. 4f, g). Up to 14 dpi with *B. bassiana, I. fumosorosea* or *L. muscarium*, the guard cells of *V. faba* showed no browning or the latter was much weaker than in *B. napus* (Fig. 4h, i, k, arrows). As in *B. napus*, no brown staining was detected in non-inoculated *V. faba* leaves (Fig. 4j). Only glands reacted with DAB, confirming the method *per se* to be effective (Fig. 6i, arrow).

Staining of B. bassiana in plants after seed coating

In faba bean seeds inoculated with blastospores of *B. bassiana* and placed on MPA, hyphae were detected up to 25 days after planting the inoculated seeds. Hyphae were seen in thin hand-cut sections in the intercellular space of the young stem by staining with solophenyl flavine 7GFE (Fig. 5b, c, arrows). When these plants were transferred into sterile soil, hyphae of *B. bassiana* could be observed up to 35 days after transplanting. They proliferated vigorously along the roots and also the shoot at a distance of

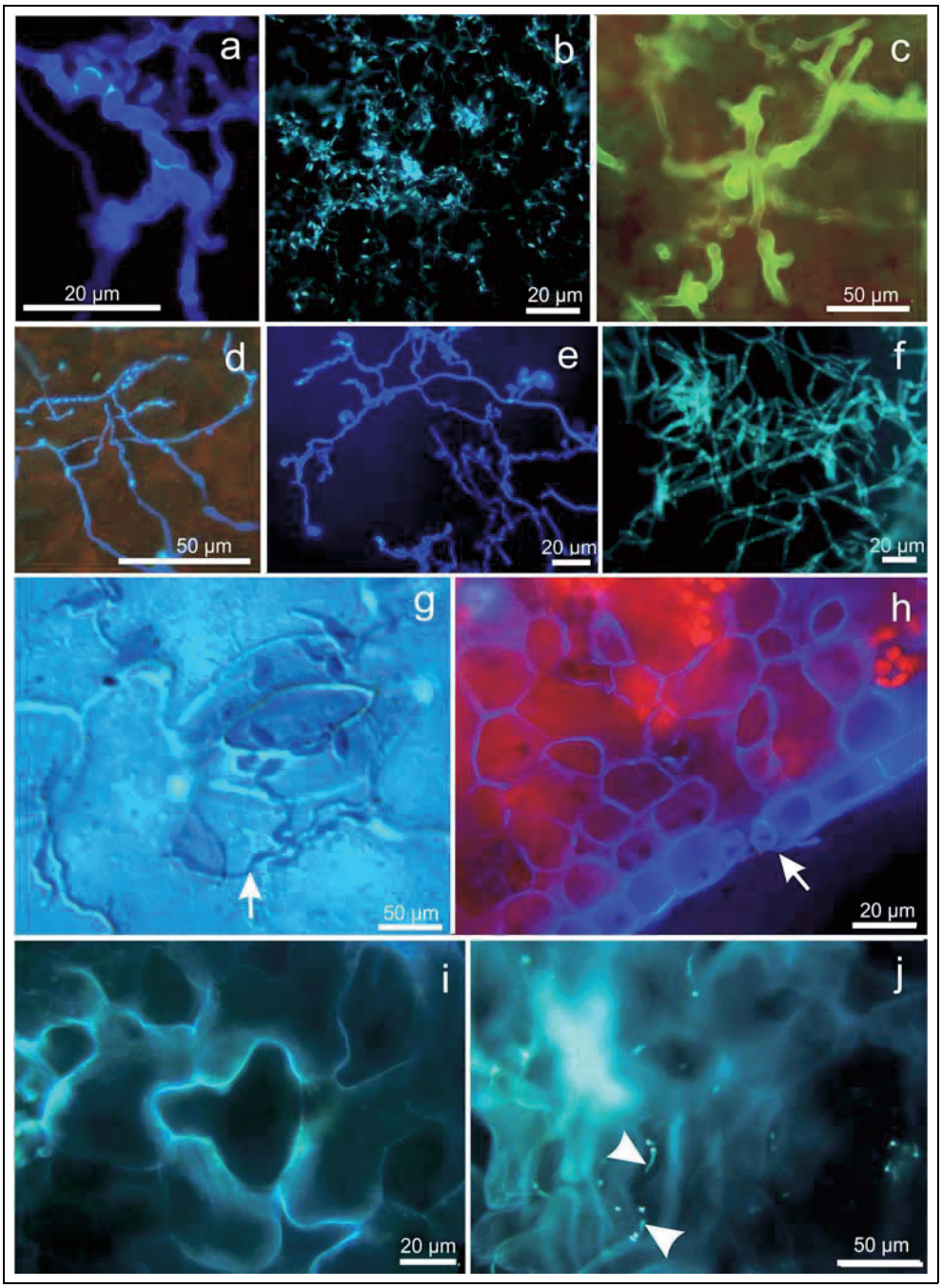

Fig. 1. Histochemical staining of hyphae of *Beauveria bassiana* and *Metarhizium anisopliae*, **a** *Beauveria bassiana* on *Brassica napus* leaves, 5 dpi, blankophor and **b, c** safranin/solophenyl 7GFE, 7 dpi, **d** *Beauveria bassiana*, still 28 dpi detectable by blankophor staining, **e** *Metarhizium anisopliae*, 3 dpi, blankophor staining and **f** safranin/solophenyl 7GFE staining, 7 dpi, **g** Germinating conidia of *Beauveria bassiana* stained with trypan blue (arrow), 6 dpi, **h** Cross section of *Brassica napus* leaf, blankophor staining, 9 dpi, no hyphae within the spongy parenchyma, but outside attached to a guard cell (arrow), **i** Control leaf of *Vicia faba* without fungal hyphae, view into the spongy parenchyma, safranin/solophenyl GFE staining after tissue clearing, **j** *Vicia faba* leaf with *Beauveria bassiana*, 2 dpi, spores and small hyphae (arrowheads) are visible in the spongy parenchyma after staining as in **i**.

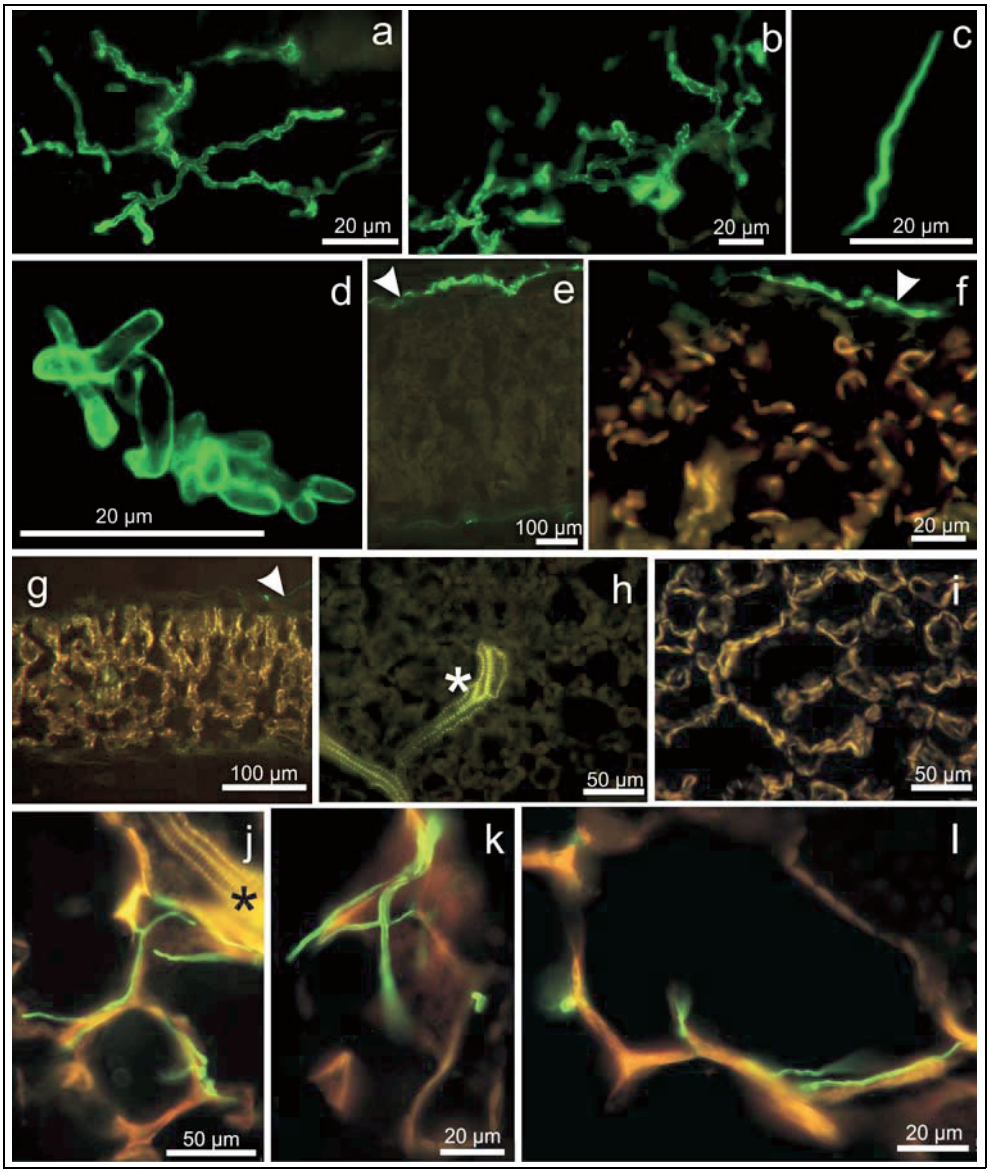

Fig. 2. Immunofluorescence labelling with rabbit primary polyclonal and FITC conjugated secondary fluorescent antibodies of *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria fumosorosea* and *Lecanicillium muscarium*, **a-f** Fungi on *Brassica napus* leaves, **a** *Beauveria bassiana* 5 dpi, **b** *Metarhizium anisopliae* 5 dpi, **c** *Lecanicillium muscarium* 2 dpi, **d** *Isaria fumosorosea* 2 dpi, **e-i** Microtome-cut cross sections (10 µm) of *Brassica napus* leaves with *Beauveria bassiana* hyphae, 5 dpi, only on the leaf surface (arrowheads), **h** Paradermal leaf section, 27 dpi, vascular bundles and distinct tracheas (asterisk) without hyphae, **i** Paradermal leaf section, no hyphae or spores inside the mesophyll, **j-l** Cross sections (10 µm) of *Vicia faba* leaves, *Beauveria bassiana,* 2 dpi, growing in the intercellular space of the mesophyll along the cell walls, except within the vascular bundle (asterisk).

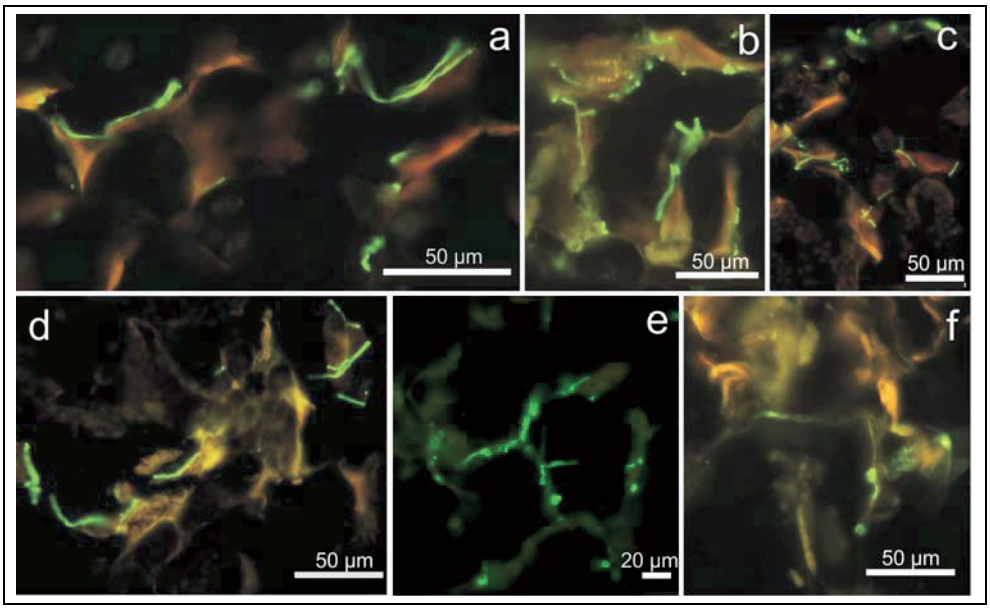

Fig. 3. Immunolocalization of fungi in the intercellular space of the mesophyll, **a, b** *Vicia faba* with *Beauveria bassiana* hyphae, 2 dpi, **c, d** *Cucumis sativus* with *Beauveria bassiana,* 2 dpi, **e, f** *Vicia faba* with *Isaria fumosorosea* hyphae, 2 dpi.

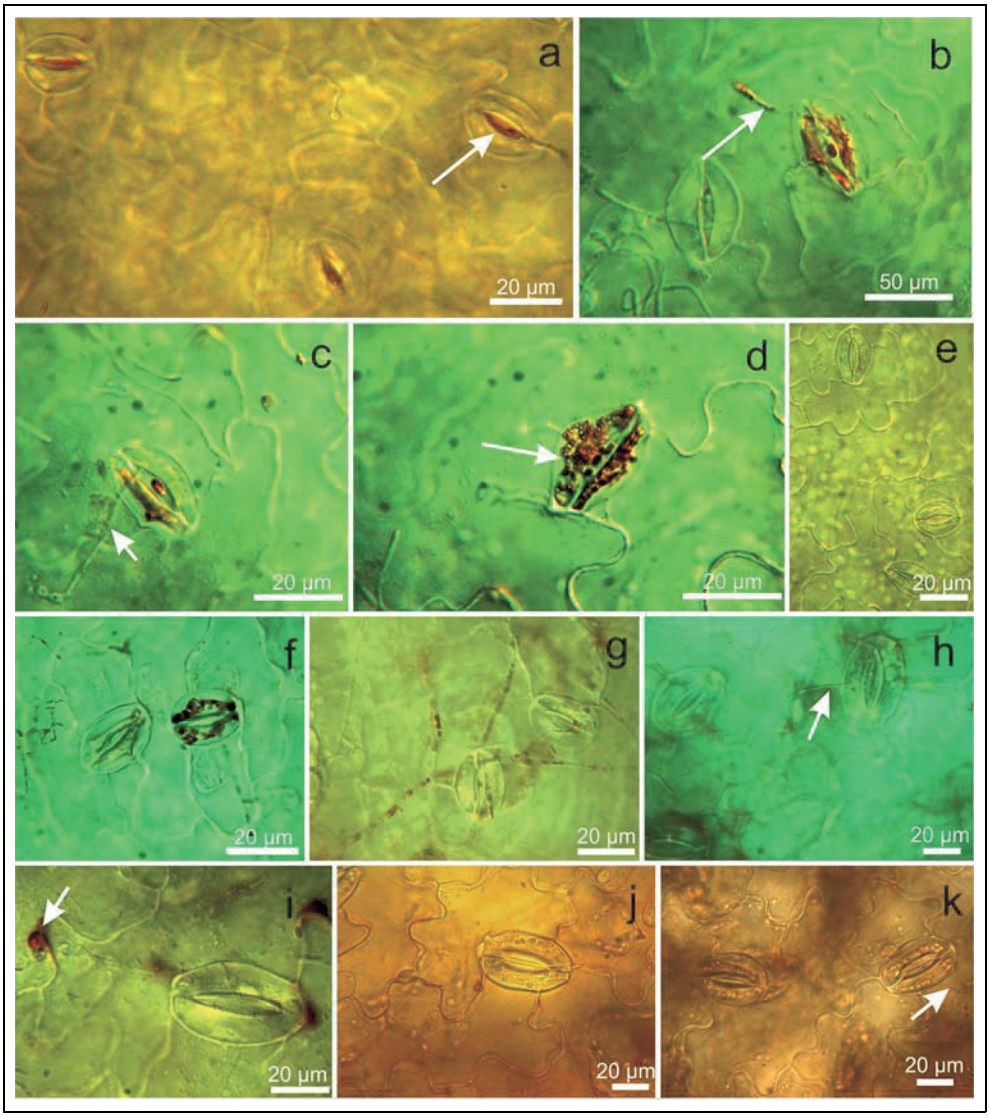

Fig. 4. Diaminobenzidine (DAB) stress test with *Beauveria bassiana, Isaria fumosorosea* and *Lecanicillium muscarium* on *Brassica napus* (**a-g**) and *Vicia faba* (**h-k**) leaves, **a** 4 hpi (arrow), **b, c, d** 1 dpi (arrows), **e** Non-inoculated control leaf, **f** *Lecanicillium muscarium*, 7 dpi, on *Brassica napus* leaf, **g** *Isaria fumosorosea*, 7 dpi, **h-k** *Vicia faba* leaves, **h** *Lecanicillium muscarium* (arrow), 7 dpi, **i** *Isaria fumosorosea*, 7 dpi, only the glands display DAB reaction (arrow), **j** Control leaf without fungal infection no brown guard cells, **k** *Beauveria bassiana* 14 dpi no DAB staining of hyphae (arrow) or guard cells.

up to about 3 cm from the cotyledons. At or within the distal stem and shoot tissue no hyphae were detected. They were predominantly found on the surface of root (Fig. 5e-g) and stem, and also within the stem epidermis and root rhizodermis (Fig. 5a) and hypodermis/protective exodermis (Fig. 5d). In particular, good growth was observed around and inside dead root hairs (Fig. 5e, f). The root cortex zone, vascular bundles and pith were free of hyphae (Fig. 5e, g, asterisks).

Re-isolation of fungi from inoculated leaves

After wound inoculation of oilseed rape cotyledons with conidia of the pathogen *P. lingam*, grey-green, non-sporulating lesions about 5 mm in diameter became visible around 7 dpi, whereas restricted dark brown lesions (1–2 mm diameter) appeared after inoculation with *P. biglobosus* (corresponding to interaction phenotypes 7 and 2, respectively, according to Mengistu et al., 1991). The only visible reaction on mock-inoculated cotyledons and on cotyledons inoculated with the entomopathogens was the formation of callus at the wounding sites, accompanied by very limited browning of the tissue in some cases (not shown). In the two experiments performed, the pathogens *P. lingam* and *P. biglobosus* could be re-isolated from all inoculation sites. The entomopathogens could also be re-isolated, but at a lower frequency. The time period between placement of the leaves on the agar until appearance of the fungal mycelium was shorter for the phytopathogens than for the entomopathogens (Table 1). Whereas *P. lingam* could be isolated also from the surrounding tissue, mycelium of *P. biglobosus* and the entomopathogens developed only at the point of inoculation (Fig. 6).

On *V. faba* wounding of leaves followed by inoculation with the pathogen, *A. fabae* resulted in the formation of a necrotic area about 5 mm in diameter. Pycnidia were not formed. Inoculation with the entomopathogens *B. bassiana, M. anisopliae* and *I. fumosorosea* led to tissue necrosis at the point of inoculation. Neither callus nor necrosis developed at mock-inoculated wounding sites (Fig. 7).

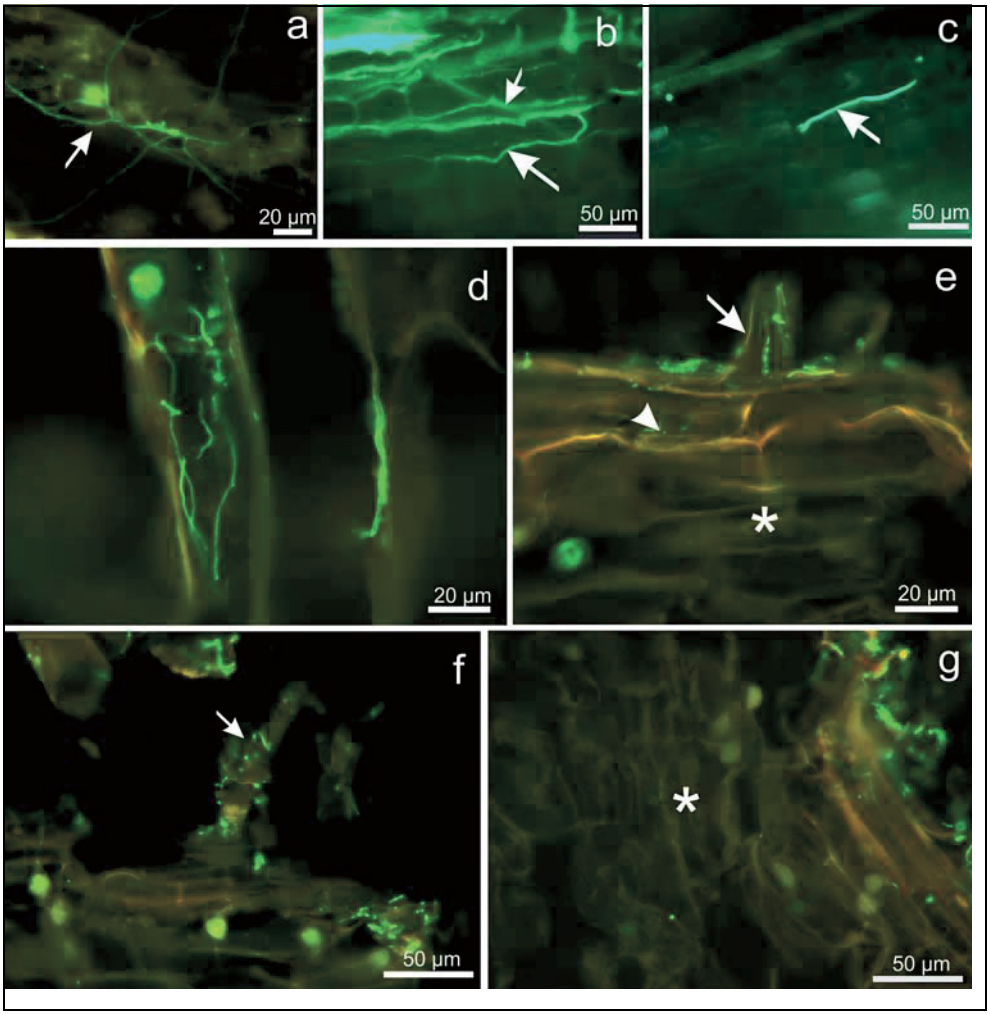

Fig. 5. Immunofluorescence detection of *Beauveria bassiana* with polyclonal rabbit primary and FITC-conjugated secondary antibodies in *Vicia faba* plants grown from seeds coated with *Beauveria bassiana* hyphae, **a** Root about 1 cm proximal to the seed, 36 dpi, abundant growth of hyphae (arrow) in the rhizosphere, **b, c** Culture of coated seeds on MPA with hyphae, 25 dpi, in the intercellular space of the seedling shoot (arrows), **d-g** Culture of coated seeds in sterile soil, 36 dpi, **d** Stem tissue with hyphae in the epidermis, **e** Hyphae at and in root hairs (arrow) and rhizodermis (arrowhead), **f, g** Hyphae only on and in the rhizodermis (arrowhead), but not within the root cortex tissue (asterisks).

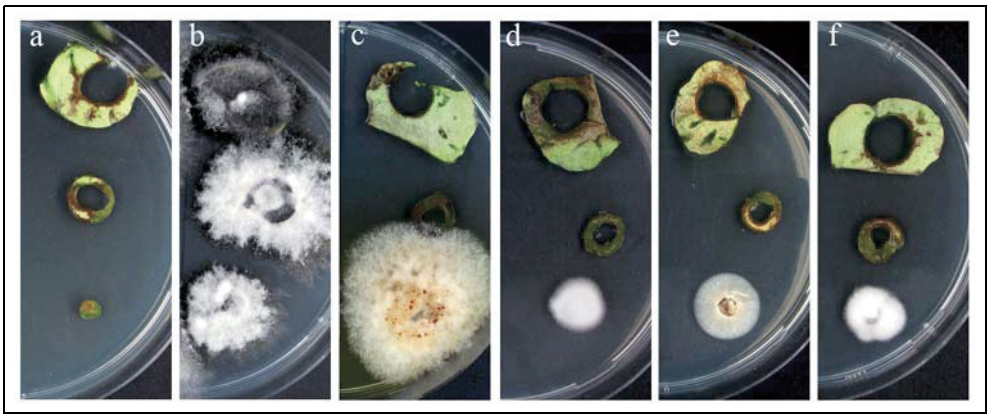

Fig. 6. Re-isolation of fungi from inoculated oilseed rape cotyledons. The cotyledons were punctured with a needle and inoculated with conidial suspensions. After 14 days they were harvested and surface sterilized. Leaf discs were excised with cork borers with different diameters and placed on PDA supplemented with antibiotics, **a** mock-inoculated, **b** *Plenodomus lingam*, **c** *Plenodomus biglobosus*, **d** *Beauveria bassiana*, **e** *Metarhizium anisopliae*, **f** *Isaria fumosorosea*.

Attempts to re-isolate the fungi 14 days after wound-inoculation were successful in the case of *A. fabae* as well as for the entomopathogens, although at a lower frequency than from the cotyledons of oilseed rape (data not shown). Two weeks after infiltration of conidial suspensions, both phytopathogens and the three entomopathogens could be successfully re-isolated from all inoculated oilseed rape cotyledons and faba bean leaves (data not shown).

Discussion

The dyes and histological methods used in the present study proved to be reliable for detection of the entomopathogenic fungi *B. bassiana, I. fumosorosea*, *L. muscarium* and *M. anisopliae*. Trypan blue, blankophor and solophenyl 7GFE had been used before for staining fungi belonging to different taxonomic groups. While trypan

Table 1. Re-isolation of plant pathogens and entomopathogenic fungi from oilseed rape cotyledons 14 days after wound-inoculation with conidial suspensions of these fungi.

	Number of re-isolations		Period until appearance of mycelium [days]	
	Experiment 1 (n = 10)*	Experiment 2 (n = 15)*	Exp. 1	Exp. 2
Mock	0	0	–	–
Plenodomus lingam	10	15	3 – 4	4
Plenodomus biglobosus	10	15	3 – 4	4
Isaria fumosorosea	7	12	6 – 7	4 – 12
Beauveria bassiana	10	12	6 – 7	6 – 15
Metarhizium anisopliae	3	13	8 – 10	4 – 12

* number of inoculation sites

Fig. 7. Symptoms on faba bean leaves present 14 days after wound-inoculation with conidial suspensions, **a** mock-inoculated, **b** *Ascochyta fabae*, **c** *Beauveria bassiana*, **d** *Metarhizium anisopliae*, **e** *Isaria fumosorosea*.

blue is a classical unspecific dye for light microscopy, the fluorophores blankophor and solophenyl flavine 7GFE are known to bind to polysaccharides (Rüchel and Schaffrinski, 1999; Wallace and Anderson, 2012). The method of combining solophenyl flavine 7GFE with pre-staining with safranin to suppress plant cell wall fluorescence (Knight and Sutherland, 2011) proved efficient also in the present experiments. To detect specific endophytes, specific antibodies were raised. Immunofluorescence microscopy with primary polyclonal and secondary FITC-conjugated antibodies also provided excellent detection of hyphae and spores of all four entomopathogens. Due to cross-reactivity, both antibodies (*i.e.* anti-*B. bassiana* and anti-*I. fumosorosea*) detected all four entomopathogens which belong to the order Hypocreales. The cross-reactivity of both purified rabbit IgGs was confirmed by plate trapped antigen ELISA (PTA-ELISA) (Rohde and Rabenstein, 2005) and western blotting experiments (WB). No reaction was observed with bovine serum albumin as negative control in PTA-ELISA. The observed immunological cross-reactivity of antibodies seem to be common in fungus serology (Kaufman and Standard, 1987; Notermans et al., 1998) including the plant invading fungi (Dewey, 2002) and suggests that several fungi share common antigenic determinants. Cross-reactivity may occur with related fungi but also, to a lesser extent, with non-related fungi (Schmechel et al., 2006; Thornton and Wills, 2015). There was no labelling of e.g. powdery mildew (data not shown).

Using light and electron microscopy, Wagner and Lewis (2000) observed germination of the conidia of *B. bassiana* on corn leaves. In some cases, hyphae developing from the spores penetrated the cuticle directly or, at a lower rate, grew through stomates into the leaves, where they branched and followed the leaf apoplast. The present studies indicated that stomates are appropriate openings for entry of spores and hyphae of all tested entomopathogenic fungi also in case of *B. napus*, *V. faba*, and *C. sativus* as the size of conidia and blastospores of the entomopathogens is significantly lower compared to the size of the stomatal openings of the tested plants.

When spores of *B. bassiana* were infiltrated into *B. napus* leaves, they appeared to germinate only on the leaf surface but not within the leaf. Nevertheless, no systemic fungal growth was observed in leaves, stems, vascular bundles or shoot apical meristems of *B. napus*. This was not only valid for *B. bassiana*, but as well for *I. fumosorosea*, *L. muscarium* and *M. anisopliae* and for all *B. napus* cultivars tested.

The experiments described here were performed with conidiospores of *M. anisopliae, I. fumosorosea, L. muscarium* and with blastospores or conidia in the case of

B. bassiana. Blastospores of entomopathogenic fungi are formed in liquid media as described in detail e.g. by Kleespies (1993), Kleespies and Zimmermann (1994), Kassa et al. (2004). They generally germinate fast and at a high rate and were therefore used in the experiments dealing with the histology of the infection process. However, in a separate study the development of *B. bassiana* on plants was identical irrespective whether blastospores or conidia were employed as inocula (unpublished results). Based on this, conidiospores were used in the present re-isolation experiments, and it is assumed that the results of the microscopical examination can be related to those of the re-isolation experiment although different spore types were used for inoculation.

The lack of systemic hyphal growth observed in the microscope studies was in agreement with the re-isolation experiments. These showed that the entomopathogens did not grow away from the point of inoculation. In this respect, they behaved differently from the pathogen *P. lingam* (teleomorph: *L. maculans*), which spread out into the adjacent tissue, and similar to *P. biglobosus* which was previously regarded as an avirulent variant of *Phoma lingam*. The re-isolation experiment further illustrated that the entomopathogens were able to persist in the tissue for at least 2 weeks, irrespective of whether they had been inoculated into wounds or infiltrated into the tissue. Since spreading fungal hyphae were not detected, the fungi must have persisted in the tissue as ungerminated spores.

The observed lack of fungal growth in *B. napus* leaves is somewhat conflicting with other reports describing Brassicaceae and *B. napus* as hosts of various fungal endophytes (Zhang et al., 2014; Card et al., 2015). From re-isolation experiments, Vidal and Jaber (2015) concluded that 12 isolates of *B. bassiana* (including isolate ATCC 74040 used in the present study) were able to colonize *B. napus* and *V. faba* leaves; two isolates could not be re-isolated in their study. Different from *B. napus*, intercellular hyphal growth of *B. bassiana*, *I. fumosorosea* and *L. muscarium* was observed in *V. faba* and cucumber leaves in the present study, best documented by detection with antibodies and enhancement by secondary FITC-conjugated antibodies. However, growth appeared to be transitional and restricted to the inoculated leaf area. When seeds of *V. faba* were coated with *B. bassiana* and placed on MPA, the parts of the shoot and root adjacent to the cotyledons became colonized with hyphae, and the latter were still present up to 35 days after transplanting into sterile soil. However, the observed hyphal growth may have been supported by the availability of nutrients from the cotyledons and may thus not have been truly endophytic. This would indicate that nutrient availability is indeed an important factor for endophytic establishment. Unequivocal detection of fungal hyphae by PCR methods may be problematic due to DNA from inocula remaining on the plant surface even after sterilization (McKinnon et al., 2017). This is supported by own observations that in spite of repeated (more than 35) washing steps with paraformaldehyde, PBS, MTSB, various ethanol concentrations and methanol, fungal spores and hyphae still remained adhered on the leaf surface (Fig. 2 e, f). Similar results have been reported for bacterial adherence on plant surfaces after disinfection or washing treatments (Fornefeld et al., 2015).

A rapid defence mechanism of plant cells is the production of reactive oxygen species (ROS), such as H_2O_2. Hydrogen peroxide is assumed to have various roles in plant defence reactions against pathogens (Lamb and Dixon, 1997) but has also been demonstrated in host-endophyte interactions (Schulz and Boyle, 2005). In the present study, browning of plant cells in the presence of fungal hyphae after treatment with DAB was much more pronounced in *B. napus* than in *V. faba* and cucumber. The response occurred already within four hours after inoculation. This indicates a direct antimicrobial activity of H_2O_2 produced by plant cells (Peng and Kuc, 1992).

To summarize, it was not possible to observe endophytic establishment of the tested entomopathogens in oilseed rape leaves, and in faba bean and cucumber colonization was restricted to the area of inoculation. We suggest this scarce colonization to be due to defence reactions like production of H_2O_2 or formation of glucosinolates (Bednarek et al., 2009), inability of the fungi to acquire nutrients in the apoplast, or a combination of both. Our results are in agreement with the general statement that endophytic colonisation by non-balansiaceous endophytes of above-ground organs in many interactions remains limited (Schulz and Boyle, 2005). However, in the case of *B. bassiana* a number of reports not only suggest movement within the plant (Ownley et al., 2008) and from soil and seeds into stems and leaves (Gurulingappa et al., 2010) but also vertical transmission via seeds (Quesada-Moraga et al., 2014). Endophytic colonization by *B. bassiana* is known to depend on the inoculation method, fungal isolate and plant species (Russo et al., 2015), however, the detection method used may also be important as was recently emphasized by McKinnon et al. (2017). From the present results it is concluded that wherever possible data on endophytic colonization should be supported by histological evidence of the kind and amount of fungal growth in the host tissue.

Acknowledgements

We thank Helga Radke, Petra Zink, Ursula Apel and Juliana Pelz for valuable technical assistance, Dr. Dietrich Stephan for providing the entomopathogenic fungal strains of JKI-Darmstadt culture collection, and Birger Koopmann, Department of Crop Sciences, Georg-August-University Göttingen, for supplying isolates of *Plenodomus lingam* and *P. biglobosus*. Many thanks for the reviewers' comments that improved the manuscript.

Dedicated to Professor Dr. Fred Klingauf on the occasion of his 80th birthday in 2016. In gratitude for his enthusiastic and very inspiring cooperation in teaching phytomedicine at the Technical University Darmstadt for many years and for his devoted supervising of the experimental

and theoretical struggle of advanced students and young scientists at the JKI Institute for Biological Control in Darmstadt.

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