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Originalarbeit

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Light microscopic studies on the development of *Beauveria bassiana* and other putative endophytes in leaf tissues

Lichtmikroskopische Untersuchungen zur Entwicklung von *Beauveria bassiana* und anderer potentieller Endophyten in Blattgewebe

Abstract

The study involved six test fungi previously recorded in the literature as being endophytes (*Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria fumosorosea, Trichoderma harzianum, Fusarium proliferatum*, *Chaetomium globosum*), two plant pathogenic fungi (*Ascochyta fabae*, *Plenodomus lingam*) and four host plants (*Vicia faba, Brassica napus, Phaseolus vulgaris, Zea mays)*. Aerial conidia, blastospores, or ascospores, respectively were applied to leaf surfaces by spraying or by infiltrating spore suspensions through stomata directly into the leaves. Observations using light microscopy showed that the test fungi germinated on the leaf surface but did not enter actively into the leaves. Within the leaves, germination of spores and growth of hyphae appeared to depend on the presence of damaged plant tissue. Various host reactions such as browning of epidermal cells and formation of papillae were observed. Colonization of healthy leaves by the test fungi in a manner similar to the pathogens *A. fabae* (on Faba bean) and *P. lingam* (on oilseed rape) was not observed. Spore germination and hyphal growth commenced when inoculated leaves were placed on agar medium. The results indicate that the test fungi possessed a saprotrophic rather than an endophytic life style when associated with leaf tissue of the studied hosts.

Key words: Fungal endophytes, entomopathogenic fungi, biocontrol, *Metarhizium anisopliae*, *Chaetomium globosum*, *Isaria fumosorosea*

Zusammenfassung

Die vorliegende Untersuchung beinhaltete sechs Testpilze, über die in der Literatur Berichte als Endophyten vorliegen (*Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria fumosorosea, Trichoderma harzianum, Fusarium proliferatum*, *Chaetomium globosum*), zwei phytopathogene Pilze (*Ascochyta fabae*, *Plenodomus lingam*) und vier Wirtspflanzen (*Vicia faba, Brassica napus, Phaseolus vulgaris, Zea mays*). Die Konidien oder Blastosporen bzw. Ascosporen der Testpilze wurden durch Sprühen auf die Blattoberfläche oder durch Infiltration durch die Spaltöffnungen appliziert. Die lichtmikroskopische Untersuchung zeigte, dass die Sporen auf der Blattoberfläche auskeimten, aber nicht aktiv in die Blätter eindrangen. Im Blattinneren schienen Sporenkeimung und Hyphenwachstum auf Bereiche mit Zell- bzw. Gewebeschädigung beschränkt zu sein. Verschiedene Wirtsreaktionen wurden beobachtet, wie die Verbräunung von Epidermiszellen und die Bildung von Papillen. Eine Besiedlung des Gewebes vergleichbar der mit den Pathogenen *A. fabae* (bei Ackerbohne) und *P. lingam* (bei Raps) wurde nicht beobachtet. Erst nach Auslegen von inokuliertem Blattmaterial auf Agarmedium setzten Sporenkeimung und Hyphenwachstum im Blattinneren ein. Die Ergebnisse deuten eher auf eine saprotrophe als auf eine endophytische Lebensweise der untersuchten Pilze im Blattgewebe der untersuchten Wirtspflanzen hin.

Stichwörter: Endophytische Pilze, entomopathogene Pilze, Biokontrolle, *Metarhizium anisopliae*, *Chaetomium globosum*, *Isaria fumosorosea*

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Introduction

Fungal endophytes comprise taxonomically distinct groups of fungi that share the ability to grow within plants during at least parts of their life cycle without causing visible symptoms (Wilson, 1995). Compared to uncolonized plants, those plants colonized by endophytes may show superior agronomic characters, such as improved growth, stress tolerance or reduced impact of insect pests or plant pathogens. Many endophytic fungi are therefore generally considered to have great economic potential not only in agronomy and plant breeding (Kuldau and Bacon, 2008; Dutta et al., 2014) but also in the biological control of plant herbivores and plant diseases (Barelli et al., 2016, Card et al., 2016, Jaber and Ownley, 2018). Probably the most advanced system in this respect is the symbiosis between pasture grasses and their *Epichloë* fungal endophytes (Johnson et al., 2013) belonging to the tribe Balansieae (family Clavicipitaceae). However, most of the endophytic fungi currently studied regarding their potential use in biological control of herbivores and plant diseases belong to the group of non-balansiaceous endophytes. Compared to the *Epichloë* grass endophytes the non-balansiaceous endophytes are far more diverse, both phylogenetically and with respect to life-history strategy (Schulz and Boyle, 2005). Due to their ability to systemically colonize their hosts, the balansiaceous endophytes are often also termed systemic endophytes, as opposed to the non-systemic and non-balansiaceous endophytes that in many interactions typically show limited colonization of above-ground plant organs (Schulz and Boyle, 2005). However, several reports suggest systemic growth also for many species of non-balansiaceous endophytes (Yan et al., 2015). According to Johnston et al. (2006), the non-grass endophytes include fungi that (1) colonise their hosts actively and extensively, (2) colonise only a small part of the leaf, (3) are isolated by host defence mechanisms but remain metabolically quiescent until host senescence, and (4) are isolated by host defence mechanisms but remain metabolically active.

Evidently, the question of systemicity of endophytic growth has practical bearing in the use of fungal biological control agents. For the entomopathogen *Beauveria bassiana* (Bals.-Criv.) Vuill., a growing body of evidence suggests the ability of this fungus to grow endophytically (Vidal and Jaber, 2015), although the methods used for assessing its endophytic nature may not have always been adequate (McKinnon et al., 2017). Many of the reports of endophytic establishment within a host are based on isolation of *B. bassiana* from sites distant from the point of inoculation, like isolation from stems, petioles or leaves after application of inocula to the host seed or soil in the plant's vicinity (Ownley et al., 2008, Akutse et al., 2013). In other experiments, whole plants or plant parts, *e.g.* leaves, were inoculated by spraying with conidial suspensions and the fungus was successfully isolated from the treated tissue after a period of incubation (Gurulingappa et al., 2010, Vidal and Jaber, 2015).

Given the increasing interest in understanding and exploiting the endophytic lifestyle of particular fungal species for their biocontrol potential, information is required on the relationship between host and endophyte at the histological level. In a previous study with *B. bassiana* and other entomopathogenic fungi, we observed germinating spores and short fungal hyphae of the entomopathogens at the inoculation sites on *Vicia faba* L. leaves but no hyphal growth extending into the leaf tissue. After inoculation of *Brassica napus* L. with the same fungi, hyphae in leaves were not seen, and host cells in contact with the fungus showed browning after treatment with 3, 3 –diaminobenzidine (DAB), indicating the production of H_2O_2 as a defence response. These microscopical observations were in agreement with experiments in which the inoculated fungi could only be isolated from the points of inoculation but not from the surrounding tissue. Overall, these observations indicated a lack of endophytic growth of the studied entomopathogens in leaves (Ullrich et al., 2017).

With the aim to substantiate these findings we modified, repeated and extended the microscopical studies. In order to prove that the staining method *per se* was suited to visualise fungal hyphae in leaf tissue and also to be able to compare the development of the potential endophytes with that of pathogens on the same host we included the fungi *Ascochyta fabae* Speg. and *Plenodomus lingam* (Tode) Höhn. (syn. *Phoma lingam* (Tode) Desm.) in our study. In addition to *Vicia faba* and *Brassica napus* that had already been included in the previous study we employed *Phaseolus vulgaris* L. and *Zea mays* L. which have both been described as hosts of *B. bassiana* (Wagner and Lewis, 2000; Parsa et al., 2013; Parsa et al., 2018). Five more fungal species previously recorded in the literature as displaying endophytic lifestyles were also studied, namely *Metarhizium anisopliae* (Metschn.) Sorokīn, *Isaria fumosorosea* Wize (previously termed *Paecilomyces fumosoroseus* (Wize) Brown and Smith), *Trichoderma harzianum* Rifai, *Chaetomium globosum* Kunze and *Fusarium proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg.

Members of the genus *Metarhizium*, including *M. anisopliae*, appear to be primarily root colonizers (Hu and St. Leger, 2002; Behie et al., 2015; Greenfield et al., 2016). However, after application to tomato roots (Krell et al., 2018) or Faba bean seeds, one species, namely *M. brunneum* was found to colonise above-ground plant tissues while improving plant growth (Jaber and Enkerli, 2016). For *M. anisopliae,* endophytic growth in corn, tomato and oilseed rape was shown by isolation of the fungus from plant organs distant from the original site of inoculation (Bing and Lewis, 1992; Garcia et al., 2011; Batta, 2013). *I. fumosorosea* is commercially used for the biocontrol of several pest insects, mainly whiteflies (Zimmermann, 2008), and has been reported to grow endophytically in leaves and stems of *Sorghum bicolor* and in roots of *Festuca arundinacea* (Mantzoukas et al., 2015, Gan et al., 2017). *F. proliferatum* is a moderately aggressive pathogen of maize, onion, wheat and other crop plants. Similar to other fusaria (Kuldau and Yates, 2000) it can survive in plants without causing visible

disease symptoms. There are a number of reports describing the isolation of this species from healthy plants (*e.g.* WANG et al., 2014, LI et al., 2015). *Trichoderma* spp. are primarily soil inhabiting fungi characterised by a high degree of saprophytic competiveness towards other fungi, including many plant pathogens. Specific strains and species have been however reported as endophytes of woody plants like *Theobroma* spp. and *Hevea* spp. (CHAVERRI et al., 2011). Effective biocontrol strains of *Trichoderma* are able to colonize the root epidermis and outer cortical layers (HARMAN et la., 2004). Endophytic colonization of above-ground plant parts following application of *Trichoderma* conidia to seeds or the soil has been reported for *Phaseolus vulgaris* (MUTUNE et al., 2016) and cabbage (ZHANG, 2014). In the latter work, one of the two strains used was *T. harzianum* T39 that was also used in our study. *T. harzianum* T39 is a biocontrol strain with several suggested modes of action including competition, restraint of the pathogen`s enzymes and induced resistance (NICOT et al., 2016). Among the large number of species (around 95 recognised species) in the ascomycete genus *Chaetomium,* most of the literature is concerned with a single species, namely *C. globosum*. Growing primarily on cellulose substrates, *C. globosum* is widespread in the environment, but often also found associated with plants. *C. globosum* is known as an endophyte of *Ginkgo biloba* where it has been isolated from leaves (QIN et al., 2009) and bark (ZHANG et al., 2013). It has, however, also been isolated from other plants and plant parts, *e.g.* leaves of *Curcuma wenyujin* (WANG et al., 2012), *Triticum aestivum* (DINGLE and MCGEE, 2003), *Altea rosea* (ABOU ALHAMED and SHEBANY, 2012) and stems of *Salvia officinalis* (DEBBAB et al., 2009). Endophytic colonization of cotton by *C. globosum* was associated with a range of negative effects on herbivores with different feeding modes (ZHOU et al., 2016). A number of reports describe effects of *C. globosum* on oomycete and other plant pathogens, making it a candidate agent for biocontrol of many plant diseases (RAGUCHANDER et al., 2014).

Two inoculation methods were used in the current study, an aqueous spray with conidia or ascospores, respectively or infiltration of an aqueous spore suspension into host leaves via stomatal openings. The rationale for using infiltration as an inoculation method was twofold. Firstly, we assumed that infiltration would enhance endophytic colonisation as it spares the fungus a critical step of entering into the leaf. Many endophytic fungi, like the *Epichloë*, lack any physical mechanism to enter directly into leaf tissues (such as via hydrolytic enzymes or infection pegs). Secondly, we speculated that due to the presence of water films on plant cell walls and the high relative humidity in the inner air spaces of the mesophyll (TAIZ and ZEIGER, 1998), fungal spores would germinate at a higher frequency than on the leaf surface. Samples were taken at different times after inoculation, and whole leaf mounts, stained with lactophenol-trypan blue, were viewed under a light microscope. In addition, fungal development was followed microscopically in pieces of inoculated leaf tissue placed on an agar medium. The results obtained confirm those of our previous study (ULLRICH et al., 2017) showing that the fungi were unable to colonize the leaves and this information indicates that this failure is related to the inability of the fungus to acquire sufficient nutrients from the host plant. The results are discussed in relation to published histological studies describing interactions between plants and non-balansiaceous endophytes.

Materials and methods

Plant material

Inoculation experiments were performed with oilseed rape (*B. napus cv*. Adriana), Faba bean (*V. faba cv*. Espresso), common bean (*P. vulgaris cv*. Maja) and maize (*Z. mays cv*. Emmy and Oberst). The plants were cultivated in 12 cm plastic pots in a standard potting substrate (Fruhstorfer Erde Typ LD 80, Hawita Gruppe GmbH, Vechta, Germany) mixed with sand (3: 1, v/v/). The experiments with Faba bean, common bean and maize were performed in a growth room at 20 °C under fluorescent tubes (16/8 h day/night). Oil seed rape seedlings were cultivated in a greenhouse (20–23°C) with additional light supplied from sodium high pressure lamps.

Fungi

The fungi used in the inoculation experiments were the entomopathogens *B. bassiana* strain ATTC 74040 (isolated from the product Naturalis®; I.R.C.A. Service S.p.A., Fornovo San Giovanni, Italy), *I. fumosorosea* strain JKI-BI-1496 and *M. anisopliae* strain JKI-BI-1339, one strain each of *C. globosum* and *F. proliferatum,* and *T. harzianum* strain T39. The strains of *C. globosum* and *F. proliferatum* and *T. harzianum* strain T39 were taken from the culture collection of the Institute for Biological Control. *T. harzianum* strain T39 was originally isolated from the biocontrol product Trichodex (Makhteshim Agan, Israel). *Plenodomus lingam* (syn. *Phoma lingam*; teleomorph *Leptosphaeria maculans* Ces. & De Not.) strain T12aD34 and a strain of *A. fabae* (kindly supplied by Birger Koopmann, Georg-August-Universität Göttingen and Mathias Hahn, Technische Universität Kaiserslautern, respectively) were included as pathogen controls.

Cultivation of fungi and inoculation of plants

The fungi were cultivated in the dark at room temperature in Petri dishes on malt extract peptone agar (MPA; 30 g malt extract, 5 g soybean peptone, 18 g agar per 1000 ml distilled water; *B. bassiana*, *I. fumosorosea, M. anisopliae*), potato dextrose agar (PDA; Sigma Aldrich; *C. globosum, T. harzianum, F. proliferatum*), or V8-agar (MILLER, 1955; *P. lingam*). For the preparation of inoculum, plates with sporulating cultures were flooded with sterile distilled water (0.0125% Tween® 20; Sigma-Aldrich) and scraped with a spatula. The resulting suspensions were filtered through cotton gauze (Mullro®) to

remove mycelial fragments and the concentration of the resulting spore suspension was measured with a haemocytometer and adjusted to 1×10^6 ascospores in the case of *C. globosum* and to 1×10^7 conidia per ml for all other fungi. Blastospores of *B. bassiana* were produced by cultivation for 5–7 days at 25°C on a rotary shaker in Czapek-Dox broth (Sigma-Aldrich) (50 ml per 300 ml flask). The cultures were then centrifuged for 15 min at 4000 rpm and the pelleted blastospores used to prepare suspensions in sterile distilled water (0.0125% Tween® 20) containing $1x10^7$ blastospores per ml.

The following plant-fungus combinations were studied: oilseed rape: *B. bassiana*, *I. fumosorosea*, *M. anisopliae*, *P. lingam*; Faba bean: *B. bassiana*, *I. fumosorosea*, *M. anisopliae*, *A. fabae*; common bean: *B. bassiana*, *F. proliferatum*, *T. harzianum*, *C. globosum*; maize: *B. bassiana*.

The plant parts inoculated were cotyledons (oilseed rape), primary leaves (common bean), leaflets of the 3rd to 5th pinnate leaves (Faba bean) or leaf blades of the 2nd or 3rd leaves (maize), respectively. The spore suspensions described above were either sprayed onto both leaf surfaces, unless stated otherwise, using a chromatographic sprayer (Ecospray, Kito Production, Aix-en-Provence, France) or infiltrated into the leaves. The latter was achieved by gently pressing the open end of a syringe (without a hypodermic needle) on the abaxial side of the leaf and applying sufficient pressure to introduce the spore suspension through the stomata into the leaf (ULLRICH et al., 2017). Aliquots of the spore suspensions used for inoculation were routinely plated on PDA, and spore germination was checked after overnight incubation. In one experiment, germination of the conidia and blastospores of *B. bassiana* was determined after plating on PDA, CzA (Czapek-Dox broth solidified with 1.8% Agar), SNA (NIRENBERG, 1976) and water agar (1.5%) prepared with distilled water.

After inoculation, the pots were incubated for 18–20 h in an unlighted humid chamber and afterwards returned to the growth room. In a few experiments they were covered with a plastic bag to increase humidity for two more days. All experiments were performed at least twice.

Fungal staining and microscopy

At different intervals after inoculation pieces of leaf tissue (1 × 1 cm) were excised and boiled for two to four min in lactophenol-trypan blue staining solution (10 ml lactic acid, 10 ml glycerol, 10 ml water, 10 g phenol, 10 mg trypan blue). They were then cleared in saturated chloral hydrate (5 g per 2 ml water) for 5–10 min, mounted in the same solution (KOCH and SLUSARENKO, 1990) and viewed under the differential interference contrast optics of a Zeiss Axioscope 2 microscope. Micrographs were taken using a digital microscope camera (Moticam 2300; Moticam, Hong Kong). In two inoculation experiments with *B. bassiana* and *C. globosum* and common bean, spores were sprayed on the lower side of the leaves, and samples were taken 5 or 9 days post inoculation (dpi) and divided in two equal portions. One was used for immediate staining and microscopic inspection. The other half were placed on PDA in Petri dishes containing 0.002% Rifampicin + 0.005% Streptomycin with the inoculated (abaxial) surface facing the agar. Before placement on agar the samples were surface disinfected by placement for 10 min in 1% NaOCl. After incubation of the Petri dishes at room temperature for 2–5 days the leaf pieces were taken from the agar, stained and inspected microscopically. Leaf pieces sampled (= 7–10 dpi) in parallel from inoculated, intact leaves (*i.e.* not placed on PDA) served as controls.

In the case of *I. fumosorosea* and *M. anisopliae*, aerial conidia, and in the case of *B. bassiana* aerial conidia and blastospores were produced as described above, plated on agar medium, and the width and length of at least 100 spores were measured using computer software of the microscope camera, and means and standard deviations were calculated.

Results

Development of the phytopathogens A. fabae and P. lingam

Infection of Faba bean by *A. fabae* was by direct penetration of epidermal cells or by growth through stomata. Infection through stomata was seen in samples taken 3 and 7 dpi and was apparently preceded by considerable growth of hyphae on the leaf surface. Single hyphae entered stomata without the formation of an appressorium (Fig. 1a, 1–6b). In other cases the infecting hyphae seen in stomatal cavities were without connection to hyphae on the leaf surface. This indicates that they originated from germinated conidia in the upper part of the stoma and that after germination they were no longer recognizable as conidia. Direct penetration occurred through epidermal cells (Fig. 2a, 2b; arrows) or between anticlinal epidermal cell walls (not shown) and was frequently accompanied by the formation of a brown pigmentation, from the host, around the penetration sites. The majority of infections of *B. napus* by *P. lingam* was through stomata. Germinating conidia gave rise to thin hyphae that grew down the stoma into the mesophyll (Fig. 4a–d). The observed few cases of direct penetration by *P. lingam* were through anticlinal walls (Fig. 5a, 5b). Once inside the leaf the hyphae of both *A. fabae* and *P. lingam* branched and colonized the host tissue intercellularly. Hyphae first spread in the space immediately below the epidermis and later also colonized the spongy mesophyll (Fig. 3b, 6). In the case of leaves already colonized by *A. fabae* or *P. lingam*, respectively, care had to be taken not to mistake infection through stomata for growth of hyphae out of stomata, which was occasionally seen.

Development of Isaria and Metarhizium

The development of *I. fumosorosea* and *M. anisopliae* was followed on both Faba bean and oilseed rape. The conidia of *I. fumosorosea* were oval and measured 3.98 ± 0.42 × 1.80 ± 0.19 µm on average, those of *M.*

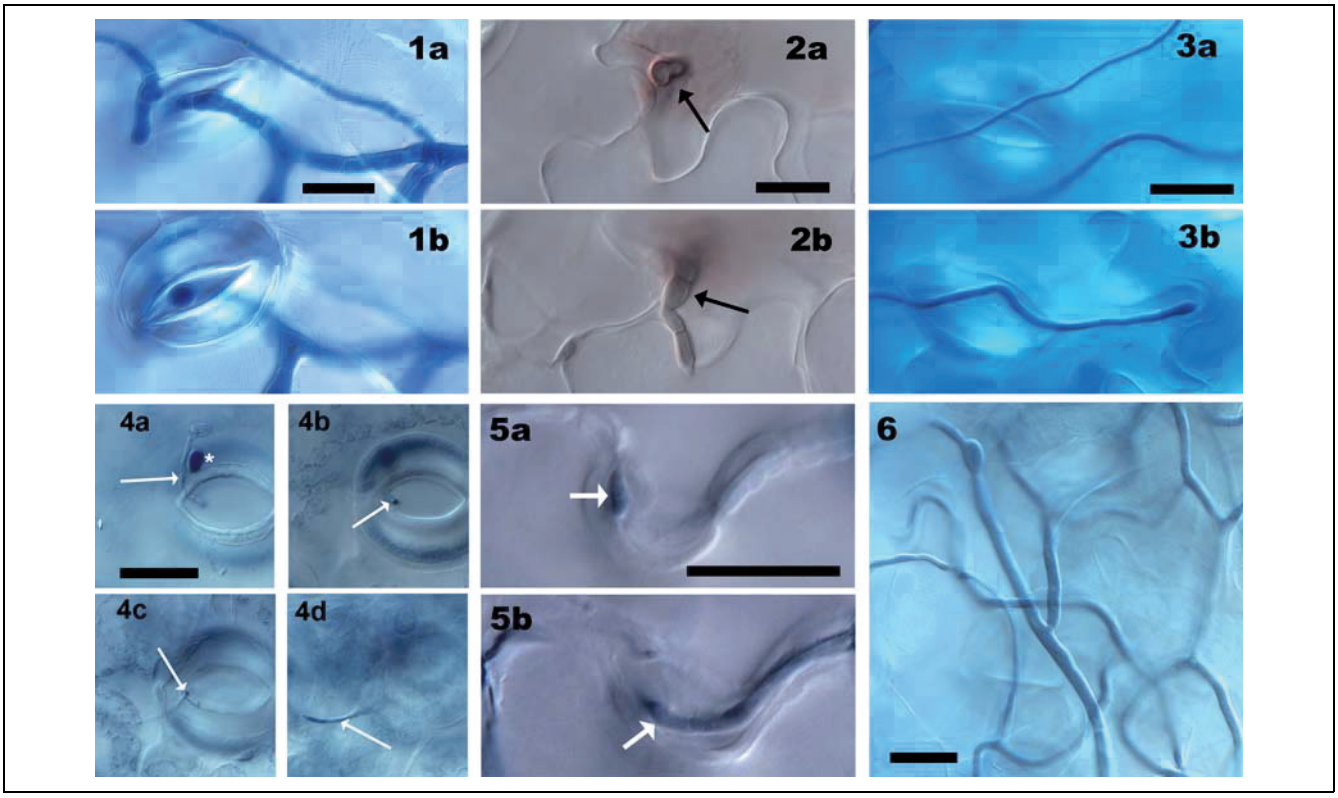

Fig. 1–6. *Ascochyta fabae* and *Plenodomus lingam* on *Vicia faba* and *Brassica napus*, respectively (scale bars = 20 µm):
1. Infection of *V. faba* via a stoma by a superficially growing hypha, same subject in different focal planes; (a) hypha entering the stoma, (b) the same hypha within the stoma
2. Infection of *V. faba* by ingress through the cuticle and epidermal cell (arrows), same subject in different focal planes; (a) hypha in epidermal cell, (b) the same hypha emerging below the epidermis
3. Hyphae of *A. fabae*, same subject in different focal planes; (a) two hyphae growing on the leaf surface, one of them over a stoma, (b) an intercellular hypha growing in the mesophyll below the same stoma
4. Infection of *B. napus* via a stoma, same subject in different focal planes; (a, b) ungerminated (asterisk) and germinated conidium with infection hypha (arrow) growing into the stomatal opening, (c, d) infection hypha (arrow) below the stoma growing into the mesophyll
5. Infection of *B. napus* by ingress between epidermal cells, same subject in different focal planes; (a) fungal hypha (arrow) growing between anticlinal walls of bordering epidermal cells, (b) the same hypha emerging below the cell wall and continuing growth below epidermal cells
6. Intercellular hyphae of *P. lingam*.

anisopliae were cylindrical and measured 6.77 ± 0.64 × 2.80 ± 0.30 µm (Fig. 7a, 7b). After inoculation by infiltration of *V. faba* the conidia of both fungi were seen above and within stomata (Fig. 8a, 8b) and in the spongy mesophyll. Some conidia of *I. fumosorosea* germinated in the mesophyll (Fig. 9a) but without developing further. In samples viewed 14 days after inoculation ungerminated conidia were still present in the spongy mesophyll (Fig. 9b). As described below for *B. bassiana*, Faba bean reacted also to the attempted ingress by *I. fumosorosea* (Fig. 10a–c). However, compared to *B. bassiana* the response was much more frequent and pronounced as judged by the brown pigmentation which appeared to be most intensive at the points of attempted penetration (Fig. 10b). Successful penetration of epidermal cell walls by *I. fumosorosea* was never observed. *M. anisopliae* was able to enter the mesophyll of *V. faba* from conidia germinating within stomata (Fig. 11a–c). The fungus also formed germ tubes and appressoria over stomata (Fig. 12a) and on epidermal cells (Fig. 12b). Both oilseed rape and Faba bean responded to the presence of conidia of *M. anisopliae*. In oilseed rape cotyledons, appositions were seen in the walls of mesophyll cells at points of contact with the conidia (Fig. 13a). In Faba bean, brown pigmented appositions were present below conidia on epidermal cells (Fig. 13b), and browning was also observed in areas of mesophyll cells contacting conidia (not shown). Germinating conidia of *M. anisopliae* in the mesophyll were only seen occasionally (Fig. 14a). As for *I. fumosorosea*, spreading hyphae were not observed, but often ungerminated conidia were seen in the mesophyll even 14 days after inoculation (Fig. 14b).

Development of F. proliferatum, T. harzianum and C. globosum

Following the inoculation of Faba bean and French bean leaves, germinating microconidia of *F. proliferatum* and conidia of *T. harzianum* were observed on the leaf surface, above and within stomata (Fig. 15, 16, 18) and in the spongy mesophyll (Fig. 19). On inoculated leaves of French bean, microconidia of *F. proliferatum* were seen to germinate within stomata and to grow into the substo-

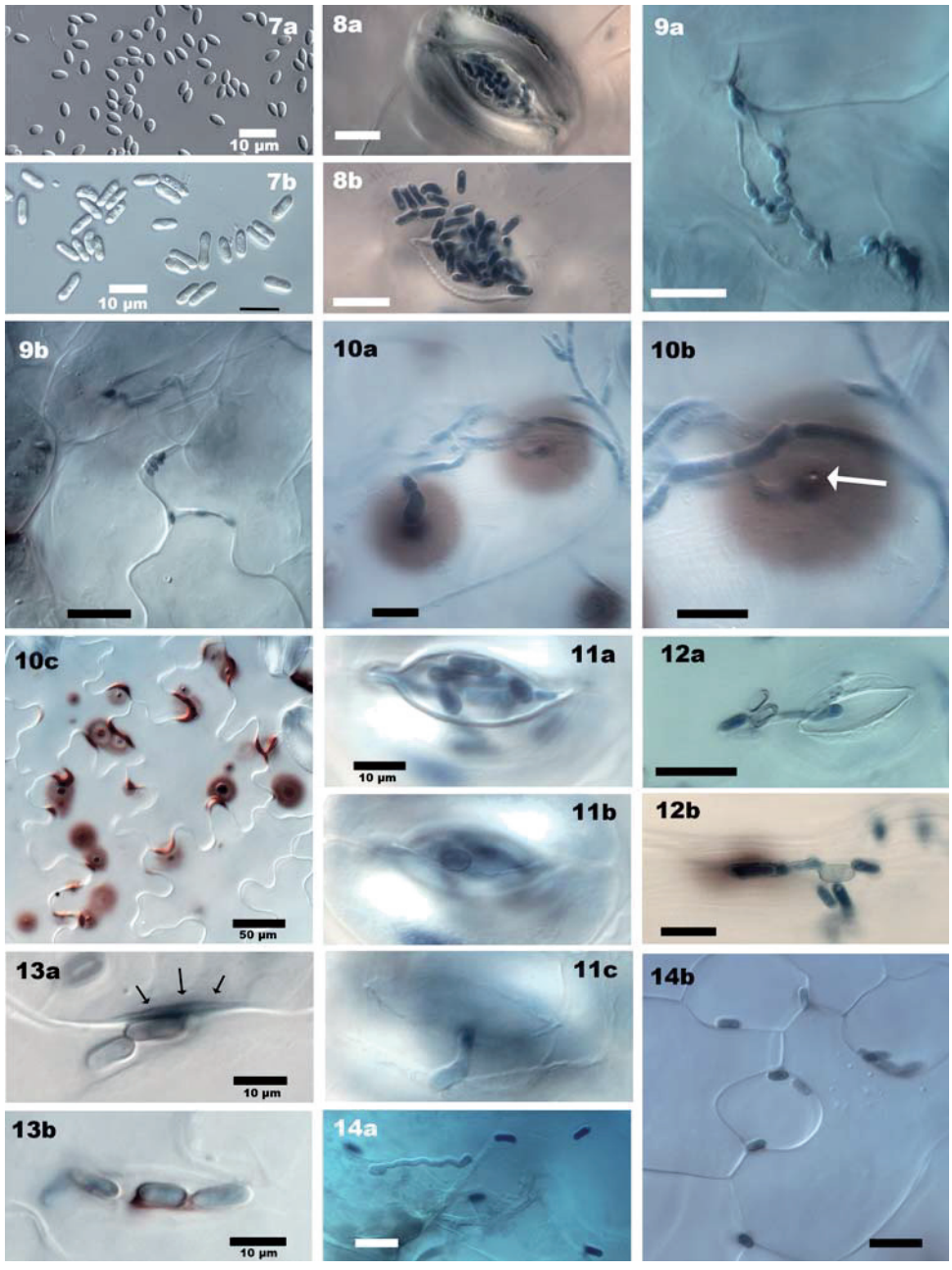

Fig. 7–14. Spores and infection structures of *Isaria fumosorosea* and *Metarhizium anisopliae* on/in leaves of *Vicia faba and Brassica napus* (scale bars = 20 µm, unless stated otherwise):
7. Ungerminated aerial conidia; (a) *I. fumosorosea*, (b) *M. anisopliae*
8. Conidia of (a) *I. fumosorosea* and (b) *M. anisopliae* within/above stomatal openings of *V. faba* (2 dpi);
9. Germinating and ungerminated aerial conidia of *I. fumosorosea* in the spongy mesophyll of *V. faba;* (a) 2 dpi; (b) 14 dpi.
10. Attempted ingress by conidia of *I. fumosorosea* into leaves of *V. faba* (7 dpi)*;* (a) browning of epidermal cells below appressoria-like structures; (b) depression in epidermal cell (arrow) at the point of attempted penetration; (c) leaf area as seen at lower magnification;
11. Conidia in of *M. anisopliae* in a stoma of *V. faba* after inoculation by infiltration, same subject in different focal planes (2 dpi); (a) ungerminated conidia in the upper part of the stoma; (b) a hypha (cross view) in the lower part of the stoma; (c) the same hypha entering the sub-stomatal cavity
12. Formation of appressorium-like structures by *M. anisopliae*; (a) over a stoma of *B. napus* (3 dpi); (b) on the surface of an epidermal cell of *V. faba* (7 dpi).
13. Reaction of host tissue in contact with ungerminated conidia of *M. anisopliae*; (a) formation of appositions (arrows) in the wall of a mesophyll cell of *B. napus* (6 dpi); (b) browning on the surface of an epidermal cell of *V. faba* (5 dpi).
14. Germinating and ungerminated conidia of *M. anisopliae* in the mesophyll of *V. faba* after inoculation by infiltration*;* (a) 2 dpi; (b) 14 dpi.

matal cavity where they branched and occasionally developed intercellular hyphae about 20–150 µm long (Fig. 16a, b). As already described for *I. fumosorosea* and *M. anisopliae*, substantial intercellular growth of *T. harzianum* and *F. proliferatum* was present only in and around wounded tissue (Fig. 17) and in pieces of leaf placed on PDA (Fig. 20).

Inoculation experiments with *C. globosum* were performed only with French bean. Due to their larger size in relation to stomata (Fig. 21a), the ascospores of *C. globosum* entered the stomatal openings less easily when inoculated by infiltration compared to the conidia of *I. fumosorosea* or *B. bassiana* (see below). Few ascospores were nevertheless seen in the mesophyll (Fig. 21b). Germination of ascospores was abundant on leaf surfaces (not shown). In the mesophyll, germinated ascospores were not observed except in damaged tissue. In the latter and after placement of inoculated leaves on PDA intercellular hyphae developed within 1–2 days. In the (not uncommon) case of individual leaf pieces lying not totally flat on the agar surface fungal development appeared to be accelerated in the parts of the leaf that were in close contact with the underlying agar, such as leaf veins, leaf margins or areas in the center of the leaf piece. A dense network of intercellular hyphae developed quickly, and after 3 to 5 days intracellular hyphae were seen in epidermal and palisade cells as well as in epidermal cells of leaf veins (Fig. 22a–c). Hyphae not only spread from the epi-

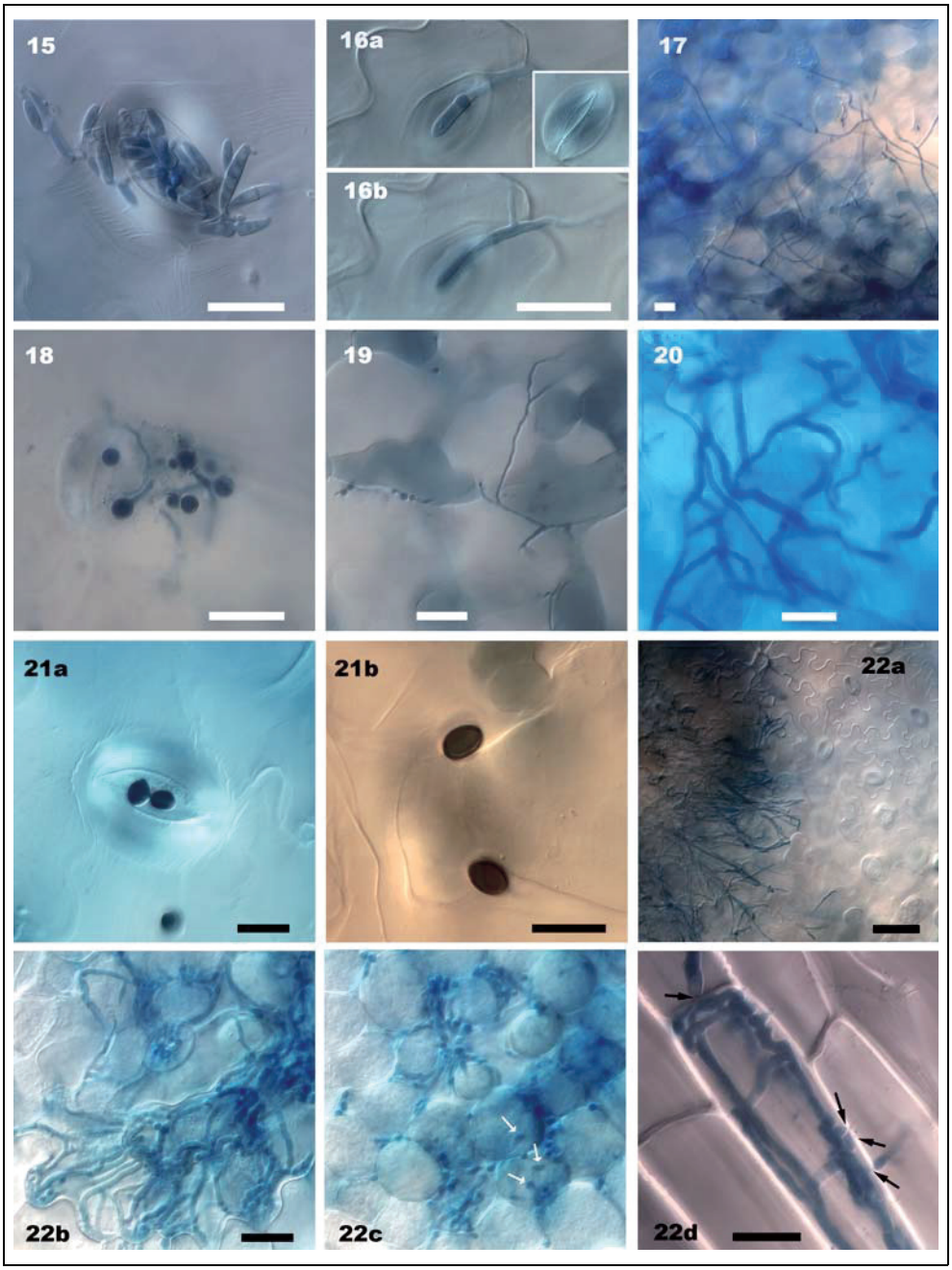

Fig. 15–22. *Fusarium proliferatum*, *Trichoderma harzianum* and *Chaetomium globosum* on *Vicia faba* and *Phaseolus vulgaris*, respectively (scale bars = 20 µm):
15. Microconidia of *F. proliferatum* over a stoma of *V. faba* 2 days after inoculation by infiltration
16. Germinating microconidium of *F. proliferatum* on *P. vulgaris* 3 days after inoculation by infiltration, same subject in different focal planes; (a) the microconidium is located within the stoma, not visible on the surface (inset), and (b) gives rise to branching hyphae in the substomatal cavity
17. Hyphae of *F. proliferatum* in damaged tissue of *P. vulgaris* (6 dpi)
18. Germinating conidia of *T. harzianum* on the leaf surface of *P. vulgaris* (4 dpi)
19. Spores and hyphae of *T. harzianum* in the spongy mesophyll of *V. faba* (3 dpi)
20. Mycelium of *T. harzianum* in the spongy mesophyll of *P. vulgaris* (8 days after placement of inoculated pieces of leaf on PDA)
21. Ascospores of *C. globosum*, (a) above a stoma, (b) in the mesophyll of *V. faba* (2 days after inoculation by infiltration)
22. Growth of *C. globosum* in leaf tissue of *P. vulgaris* placed on PDA. (a) colonization of healthy tissue by hyphae originating from the damaged area at the inoculation site. (b) intracellular hyphae in epidermal cells; (c) hyphae growing between palisade cells and in the cell lumen (arrows) (same subject as (b) in lower focal plane); (d) hyphae in epidermal cells of leaf vein spreading into neighbouring cell (arrows)

dermal cells into the mesophyll but also through cell walls from cell to cell (Fig. 22d). Occasionally, hyphae developing from germinated ascospores on the leaf surface entered into epidermal cells through the cuticle (not shown) but this was only seen in leaf samples left for several days on PDA.

Development of B. bassiana

The spores used for inoculation with *B. bassiana* were aerial conidia (Fig. 23) formed on MPA or blastospores from shake cultures in Czapek medium. The blastospores (Fig. 24) were irregularly shaped, mostly oblong to oval, measuring 6.25 ± 1.11 × 3.44 ± 0.63 µm on average. The conidia were globose to subglobose and measured 3.23 ± 0.43 × 2.81 ± 0.33 µm on average. When conidia and blastospores were plated on agar media and incubated at room temperature, germination occurred overnight at a rate of 100%, irrespective of the type of medium (PDA, Cz, SNA or WA). However, on PDA germ tube growth appeared somewhat advanced compared to water agar. Both spore types germinated with one or two germ tubes (Fig. 25). Following inoculation of Faba bean and oilseed rape by infiltration with conidia or blastospores the stomata appeared densely packed with (generally ungerminated) spores (Fig. 26, 31a). Spores were also observed in the substomatal cavity (Fig. 31b), in the space immediately below epidermal cells (Fig. 28) and in the spongy mesophyll. In

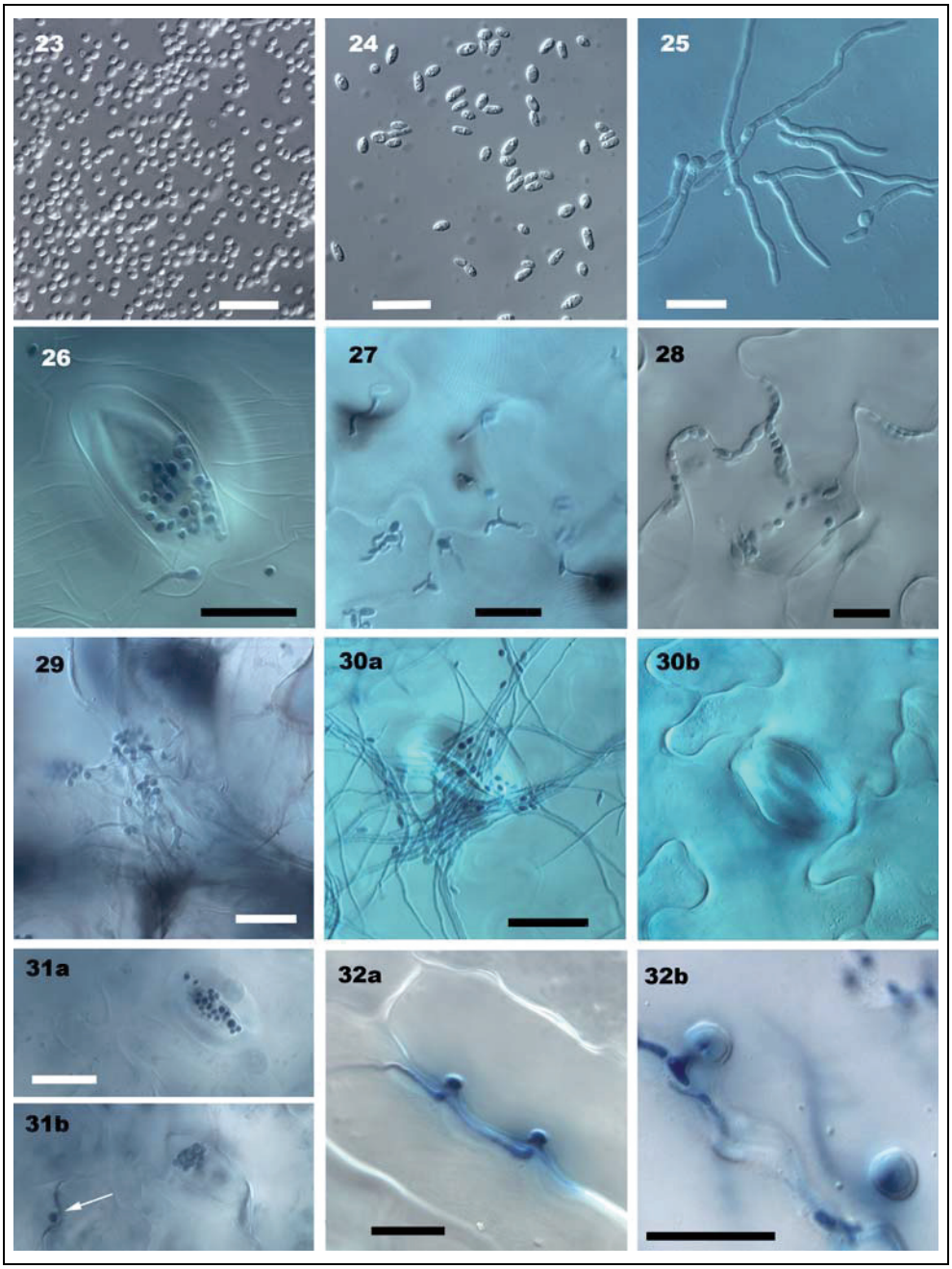

Fig. 23–32. Spores and infection structures of *Beauveria bassiana* on/in leaves of *Vicia faba*, *Brassica napus* and *Zea mays* (scale bars = 20 µm):
23. Conidiospores
24. Blastospores
25. Germinating blastospores on PDA (20 hpi)
26. Aerial conidia over a stoma of *V. faba* (2 dpi)
27. Germination of blastospores and formation of appressoria-like swellings on *V. faba* (4 dpi). Epidermal cells react with browning to the attempted ingress
28. Ungerminated and germinating blastospores below epidermal cells of *V. faba* (2 dpi)
29. Formation of hyphae by germinating blastospores in wounded mesophyll tissue of *V. faba* (6 dpi)
30. Failure of growth into a stoma of *V. faba*, same subject in different focal planes (3 dpi); (a) hyphae of *B. bassiana* growing over a stoma, (b) empty interior space of the same stoma
31. Blastospores after infiltration into *B. napus*, (4 dpi), same subject in different focal planes; (a) blastospores within the stomatal opening, (b) blastospores below the same stoma and in the mesophyll (arrow)
32. Reaction of *Z. mays* to inoculation with blastospores (5 dpi). (a) formation of cell wall appositions and (b) papillae.

leaves of Faba bean, but not in oilseed rape cotyledons, some of the spores were observed to germinate. On and in leaves, blastospores of *B. bassiana* appeared to germinate more readily than the conidia. In some instances spore germination was more extensive (Fig. 29), but this was always restricted to areas damaged during inoculation. Altogether there was no indication that spores germinating in the mesophyll developed into intercellular hyphae that spread into healthy tissue comparable to the hyphae of *A. fabae* and *P. lingam*. Furthermore, after inoculation by infiltration, many spores were still observed as ungerminated in the mesophyll at the later observation dates. In experiments with extended incubation of spray-inoculated Faba bean plants under moist conditions the spores germinated and appressoria-like structures developed at the germ tube tips. Browning of the epidermal cells in close vicinity of these structures indicated a host response to attempted penetration (Fig. 27). In some cases moist incubation resulted in the formation of mycelium on the leaf surface. However, growth of the fungus from mycelial mats formed directly over stomata into the stomatal opening and spread in the mesophyll was never observed (Fig. 30a, b). Following inoculation of maize leaves with blastospores of *B. bassiana* and moist incubation, the majority of spores remained ungerminated. Some of those that germinated developed appressoria-like swellings of the germ tube tips, preferentially over anticlinal walls of epidermal

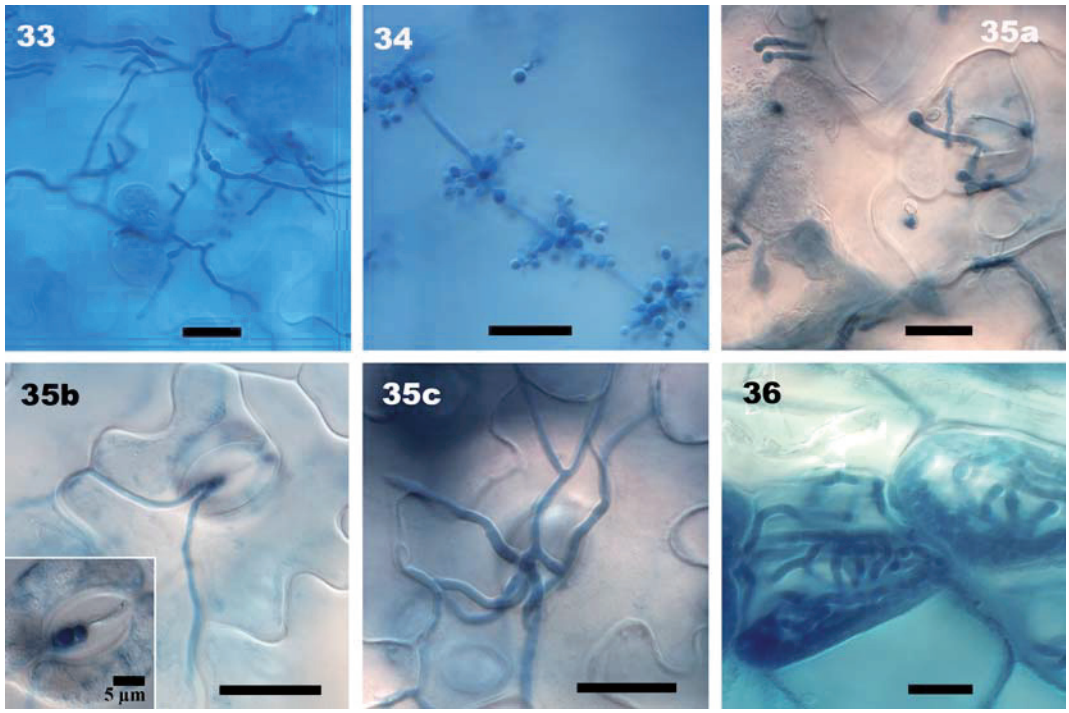

Fig. 33–36. *Beauveria bassiana* **in wounded tissue and in pieces of leaf of** *Phaseolus vulgaris* **placed on potato dextrose agar (PDA) (scale bars = 20 µm, unless stated otherwise):**
33. Profuse hyphal growth in necrotic leaf areas adjacent to infiltration sites (8 dpi)
34. Formation of conidiophores and aerial conidia over wounded tissue (4 dpi)
35. Development of intercellular mycelia. Primary leaves were spray-inoculated with blastospores, and 5 dpi pieces of leaf were placed for 2 days on PDA; (a) germinating spores in the mesophyll, (b) two hyphae originating from within the stoma (inset) and entering the substomatal cavity (same subject in different focal planes); (c) branching of hyphae and development of a mycelium in the substomatal cavity
36. Hyphae in epidermal cells of leaf veins. Primary leaves were spray-inoculated with blastospores, and 9 dpi pieces of leaf were placed for 2 days on PDA

cells. Occasionally cell wall appositions and thick papilla were seen below these structures, indicating a defence reaction by the plant (Fig. 32a, b). On both maize cultivars employed, infection of leaves through stomata, the cuticle or by penetration through anticlinal cell walls was not observed.

On French bean the early development of *B. bassiana* was similar to that on Faba bean. Following inoculation by infiltration, conidia and blastospores germinated on the leaf surface and in the mesophyll, but in undamaged tissue there was no further development. However, wounded tissue of French bean appeared to be very conducive for mycelial growth of *B. bassiana* (Fig. 33). When plants were incubated under moist conditions after inoculation, phialides and aerial conidia of *B. bassiana* developed in damaged tissue in less than 24 hours (Fig. 34), and the colonized epidermal cells in the damaged area appeared densely filled with hyphae (not shown). Microscopical observation of leaf pieces sampled five or nine days after inoculation showed that conidia or blastospores that had infiltrated into the tissue were still ungerminated, and no hyphae were present in the mesophyll. However, spore germination occurred within three to five days after placement of these leaf pieces on PDA (Fig. 35a). After spray inoculation of French bean with blastospores or conidia of *B. bassiana*, both spore types were only observed on the leaf surface. Their presence within stomata was largely obscured, but they were absent in substomatal cavities and in the mesophyll even after spray-inoculation of the leaves with an elevated spore concentration (10^8 conidia per ml). When samples from spray-inoculated leaves were placed for two days on PDA, substantial fungal growth was present on the leaf surface. Also, hyphae originating from within stomata were seen growing into the substomatal cavity (Fig. 35b) and gave rise to the development of intercellular mycelia (Fig. 35c). After three more days of incubation of the leaf pieces on PDA the mesophyll was filled with intercellular hyphae, and during the following days fungal mycelium growing out of the specimen onto the agar was clearly identified as *B. bassiana*. As already described for *C. globosum* above, fungal development and outgrowth of mycelia appeared to be fastest in the parts of the leaf that were in close contact with the underlying agar. No hyphal growth was detected in the controls (*i.e.* samples taken 9–15 dpi from leaves inoculated the same day but left on the plant during the whole period).

Discussion

Whereas there is a clear perception of the growth of the *Epichloë* endophytes in the host tissue (VOISEY, 2010; ZHANG et al., 2017), knowledge of the host-fungus interaction at the histological level of non-grass endophytes in general and entomopathogenic fungi in particular is still fragmentary. The current microscopical study was initiated to address the question of whether these fungi possess endophytic growth in plant leaves. To prove the principle suitability of the staining procedure we inoculated Faba bean and oilseed rape with conidia of the pathogens *A. fabae* and *P. lingam*, respectively. For both fungi, conidia on the host surface and hyphae in the mesophyll were readily stained by lactophenol-trypan blue. On both hosts, infection was either by direct penetration of the epidermis or by growth through stomata, which is largely in agreement with the literature. Direct infection through the epidermis by *A. fabae* has been reported before by PRITCHARD et al. (1989) and MAURIN and TIVOLI (1992). Infection through stomata, as observed in the present study, was not mentioned by these authors but has been reported for *A. rabiei* on chickpea (ILARSLAN and DOLAR, 2002). In addition to stomatal penetration by *P. lingam*, which has also been described by other workers (ABADIE and BOUDART, 1982; HAMMOND et al., 1985), we observed penetration of the cuticle and ingress through anticlinal epidermal cell walls. Direct penetration has been reported also for two other species of *Phoma*, *P. macdonaldii* on sunflower (ROUSTAEE et al., 2000) and *P. clematidina* on clematis (VAN DE GRAAF et al., 2002).

As inoculum of *B. bassiana* we used conidia and blastospores, respectively. In nature, blastospores are formed solely in infected insects and are therefore not considered to be involved in the spread of the fungus in the environment. Regarding the interaction with the plant we observed no principle differences between the two spore types except that in the inoculation experiments the more voluminous, physiologically active blastospores tended to germinate more regularly and form larger hyphae than the conidia. Endophytic colonization of maize plants by *B. bassiana* has been reported after seed inoculation and foliar sprays with conidia at the whorl stage (BING and LEWIS, 1991; RAMIREZ-RODRIGUEZ and SÁNCHEZ-PEÑA, 2016). Although in the present study maize plants were spray-inoculated with blastospores or conidia at a comparable plant developmental stage and incubated under comparable conditions, infection by *B. bassiana* was not observed. Occasionally, appressorium-like structures were formed, but infections were apparently warded off by thick papillae and appositions of host material formed at the attempted sites of penetration, which may indicate a mismatch between the maize variety and *B. bassiana* strain used.

Host reactions were recorded not only in maize but also in Faba bean leaves and oilseed rape cotyledons. In the latter, cell wall appositions were occasionally observed at the points where conidia of *M. anisopliae* contacted mesophyll cells. Appositions of host material were also present in anticlinal cell walls, surrounding the infection hyphae of *P. lingam*. The typical host reaction of Faba bean were brown pigmented, often halo-shaped areas and papillae at the sites of attempted penetration. They were present particularly below appressoria of *M. anisopliae* but also formed in response to *I. fumosorosea* and *B. bassiana* and the pathogen *A. fabae*. The formation of appressoria by *M. anisopliae* on the plant surface is in line with experiments showing that they are produced on a wide range of hard, preferably hydrophobic surfaces (ST. LEGER et al., 1989). The germination rate on leaf surfaces of the conidia, blastospores or ascospores, respectively of all putative fungal endophytes employed varied between experiments. Other than for the pathogens *A. fabae* and *P. lingam,* direct penetration of epidermal cells of intact leaves, *i.e.* leaves attached to plants, by these fungi was not observed.

Infiltration of spore suspensions into leaves failed in the case of maize but was possible and applied for inoculation of oilseed rape, Faba bean and common bean. The ease of successfully infiltrating the spore suspensions into the leaves was related to spore size; it was readily achieved with the small sized conidia of *I. fumosorosea*, *B. bassiana*, *T. harzianum* and *M. anisopliae* but difficult with the much larger ascospores of *C. globosum*. Blastospores, conidia and ascospores, respectively, of all potential endophytes germinated at a high degree on PDA or water agar (the latter was tested only for *B. bassiana*), whereas after infiltration into leaves, germinating spores were either not seen, as with *B. bassiana* in oilseed rape cotyledons, or observed only occasionally. In the spongy mesophyll of Faba bean, ungerminated, visibly intact conidia of *M. anisopliae* were present even 14 days after infiltration. In undamaged host tissue, intercellular hyphae originating from germinating spores and colonizing the mesophyll in a manner comparable to the pathogens *A. fabae* and *P. lingam* were not seen, irrespective of whether the spores germinated on leaf surfaces, in stomata or in the mesophyll. This was true for the three entomopathogens as well as for *T. harzianum*, *F. proliferatum* and *C. globosum*. Nevertheless, following inoculation into wounds *B. bassiana, M. anisopliae* and *I. fumosorosea* were shown to survive at the points of inoculation for at least 14 days (ULLRICH et al., 2017).

Despite careful handling, the host tissue was easily damaged during infiltration of spores at the sites where the open end of the syringe was pressed towards the lower leaf surface. Macroscopically these areas appeared healthy, but at the histological level damaged cells were stained a in deeper blue compared to the surrounding cells and their content often appeared granular compared to undamaged cells. In such areas, spore germination rates were higher and hyphal growth was clearly more extensive than in undamaged tissue, indicating that fungal growth depended largely on the availability of nutrients. In view of these observations, results of inoculation studies with candidate endophytes using tissue wounding should be interpreted with care.

When leaves of common bean previously inoculated with blastospores of *B. bassiana* were placed on PDA, blastospores were seen to germinate in the space between the epidermis and spongy mesophyll, and fungal hyphae originating from within stomata were present in substomatal cavities. The effect of leaf placement on PDA was even more drastic with common bean previously inoculated with *C. globosum*: while no internal fungal growth was visible prior to placement on PDA, the mesophyll became colonized in a very short time, and intracellular hyphae developed in epidermal cells. We speculate that high nutrient availability was responsible for the extensive fungal growth observed in damaged tissue and for the intra- and intercellular hyphae located in leaf tissue that was in direct contact with the underlying agar medium. We also suggest that in addition to nutrient supply other, possibly senescence-related factors contributed to the triggering of spore germination and subsequent mycelial growth when detached leaves were placed on PDA.

The diversity of infection patterns of endophytic fungi is reflected in the work by CABRAL et al. (1993) who studied the cytology of the interactions between one annual and three perennial *Juncus* species and five different fungal endophytes. The infection types observed ranged from colonization of a single host epidermal cell to the formation of extensive intra- and intercellular hyphal networks. Colonization by *Alternaria alternata* and *Cladosporium cladosporioides* was confined to the immediate substomatal area. The authors assumed that the colonization pattern observed was that characterised by opportunistic saprophytes, and that the localization within the stomatal chamber may provide protection against compounds used for surface disinfection (*e.g.* ethanol and sodium hypochlorite). After inoculation by infiltration, germination of conidia in substomatal cavities and adjacent tissues was also seen in our study but generally suspected to have been triggered or at least promoted by nutrients leaking from damaged tissue. The observation that in French bean leaves placed on PDA hyphae entering the substomatal cavity appeared to emerge from within stomata strongly indicated that spores were able to persist within the stomata. Interestingly, we saw the same pattern of colonization in leaves spray-inoculated with conidia of *B. bassiana* and surface-sterilized with 1% NaOCl before placement on agar, indicating that not only the substomatal chamber but also the space within stomata provides protection against some sterilants (unpublished results).

Taken together, the results presented here confirm those of our previous study (ULLRICH et al., 2017) that failed to show systemic endophytic growth of the inoculated entomopathogenic fungi in leaves of different host plants. In addition, the present results indicate a saprotrophic rather than an endophytic life style of the fungi studied. This may be less surprising for *C. globosum, T. harzianum, F. proliferatum* and *I. fumosorosea* that to our knowledge have not been reported as endophytes in leaves of Faba bean, oilseed rape and French bean before. It is, however, somewhat unexpected for *M. anisopliae* and especially for *B. bassiana* (including strain ATTC 74040, Naturalis®, used in the present study) that has been reported as an endophyte of many crops (VIDAL and JABER, 2015). Some of these reports provide histological evidence for the endophytic colonization. For maize the histology of the infection process has been described in detail based on light-, scanning- and transmission electron-microscopical examination of leaves from young plants (WAGNER and LEWIS, 2000). After inoculation of leaf petioles of date palm by wounding, *B. bassiana* extensively colonized parenchyma cells inter- and intracellularly and was also present in vascular tissue (GÓMEZ-VIDAL et al., 2006). Colonization of palisade parenchyma and mesophyll leaf tissues by *B. bassiana* was also reported for cotton (OWNLEY et al., 2008). Growth of *B. bassiana* into poppy leaves was through the cuticle (QUESADA-MORAGA et al., 2006). Use of a GFP-tagged transformant demonstrated that *B. bassiana* effectively colonized the leaf surface of opium poppy plants during the first two days after inoculation. However, fungal colonization of the inner leaf tissues was scarce, not uniform, limited to the spaces among cells and declined even more during the following days, although fungal structures still remained present in the leaf tissue (LANDA et al., 2013). Thus, as in our study, durable endophytic growth was not observed.

The reason for the inability of the tested fungi in our study to grow endophytically is not known. It cannot be ruled out that the use of specific combinations of fungal strains and host cultivars may have given other results. Dependence of the endophytic colonization on the *B. bassiana* strain used for inoculation was observed after application to seeds of Faba bean and French bean (AKUTSE et al., 2013) and after spray application to leaves of oilseed rape and Faba bean, respectively (VIDAL and JABER, 2015). In both studies, however, the presence of the endophyte was not proven microscopically but indirectly by placement on agar medium. Most of the reports on endophytic growth of entomopathogenic fungi refer to colonization of roots and shoots after inoculation of the seeds or the soil. In view of the differences in anatomy and physiology it is obvious that results of endophytic colonization of one plant organ cannot be simply extrapolated to another. Inability of a given fungal strain to grow endophytically in leaf tissue does therefore not exclude *per se* the ability to colonize the root or stem of the same plant. However, to our knowledge this topic has rarely been addressed. Overall it appears that the interactions between endophytic entomopathogens and other putative endophytic fungi especially in leaf tissues need to be better understood before they can be exploited economically.

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