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Micron 37 (2006) 624–632
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Endophytic colonization of date palm (*Phoenix dactylifera* L.) leaves by entomopathogenic fungi

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Received 13 January 2006; received in revised form 11 February 2006; accepted 13 February 2006

Abstract

Light and scanning electron microscopy together with fungal isolation techniques were used to detect entomopathogenic fungi within young and adult date palm (*Phoenix dactylifera*) petioles and to assess fungal survival in leaf tissues. The entomopathogenic fungi *Beauveria bassiana*, *Lecanicillium dimorphum* and *Lecanicillium* c.f. *psalliotae* survived inside leaf tissues at least 30 days after inoculation. Entomopathogenic fungi colonized inoculated petioles endophytically and were recovered up to 3 cm from the inoculation site. Fungi were detected inside the parenchyma and sparsely within vascular tissue using microscopy techniques. Our results show that the entomopathogenic fungi used in this study survived and colonized date palm tissues in bioassays both under laboratory and field experimental conditions with no evidence of significant damage.

Keywords: *Phoenix dactylifera*; *Beauveria bassiana*; *Lecanicillium dimorphum*; *Lecanicillium* c.f. *psalliotae*; Entomopathogenic fungi; Bioassays; Endophytes; Scanning electron microscopy; Light microscopy

1. Introduction

Endophytic microorganisms colonize the interior of plants, especially leaves, branches, stems and roots showing no apparent harm to the host, and the interaction involves metabolic exchange between endophyte and host. They may play an important role in host protection against pests and pathogens (Lodge et al., 1996; Azevedo et al., 2000; Schulz et al., 2002). Entomopathogenic fungi can be inoculated in plants for insect control. For instance, *Beauveria bassiana* inoculated in corn leaves (Bing and Lewis, 1991, 1992a, 1992b) grew endophytically and controlled the European corn borer *Ostrinia nubilalis* (Wagner and Lewis, 2000). Fungal endophytes can also induce plant resistance mechanisms, e.g. mycotoxin production, mainly alkaloids. Protection from herbivores by fungal endophytes is best documented in agronomically important grasses, like tall fescue, in which the fungi can provide constitutive resistance through alkaloid production that is independent of herbivore damage (Bultman and Conard, 1998; Bultman et al., 2004).

The red scale insect (*Phoenicococcus marlatti*) is a very important pest of date palms (*Phoenix dactylifera* L.) in the east of the Alicante province (SE Spain) and in palm plantations of Northern Africa. Asensio et al. (2005) evaluated the parasitism of the red scale insect by the entomopathogenic fungi *B. bassiana*, *Lecanicillium dimorphum* and *Lecanicillium* c.f. *psalliotae* using microscopy techniques. They found that *L. dimorphum* and *L.* c.f. *psalliotae* developed on plant material and on scale insects forming infection structures. *B. bassiana* was found to be a weak colonizer of date palm leaves and did not parasitize the scale insects because of the tough outer wax layer of *P. marlattii* and of the abundant growth of saprotrophic fungi.

In this paper we describe the endophytic behaviour of three entomopathogenic fungi (*B. bassiana*, *L. dimorphum* and *L.* c.f. *psalliotae*) in date palm leaves using laboratory and field bioassays, as well as light and scanning electron microscopy techniques. The aim was to investigate if the fungi could grow and survive in this environment and thus be able to act as potential biocontrol agents of date palm pests and diseases.

2. Materials and methods

2.1. Collection of plant material and fungal inoculation of plants

2.1.1. Detached petioles

P. dactylifera leaves were collected from the Viveros Huerto del Cura S.A. plantations in Partida de Algorós (Elx, Alicante

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doi:10.1016/j.micron.2006.02.003

province) and from the Alicante University campus (Sant Vicent del Raspeig, SE Spain). Apparently healthy peripheral leaves from young (1.5–2.0 m high, <5-year-old) and adult (2.5–3.0 m high, ca. 15-year-old) palms were cut. After removal of limb and spines from the leaves, the petioles were placed in plastic bags and stored at 4 °C until used (ca. 3 h).

Sixty petioles (3–6 per treatment) were cut into 12 cm long pieces before processing. They were then washed with tap water and a few drops of commercial detergent (5–10 min), rinsed in distilled water (5 min) and transferred to a horizontal air flow bench for surface sterilization with 70% (v/v) and 96% (v/v) ethanol (5 min each) and air-dried.

Each surface-sterilized petiole was inoculated with 10^6 conidia of either *B. bassiana* (isolate 119, from infected coleopteran *Langia* sp., isolate 33, from infected lepidopteran *Thaumetopoea pityocampa*) or *L. dimorphum* (isolate 191, from infected *Saissetia oleae*) in 50 or 100 μl of 0.02% Tween-20, from 20-day-old colonies on CMA. All fungi belong to our laboratory collection and are kept at 4 °C in the dark on corn meal agar (CMA, from BBL). Controls (uninoculated petioles) were prepared with 0.02% Tween-20 in distilled water.

P. dactylifera petioles were transversely wounded with a sterilized razorblade and inoculated with the corresponding conidial suspension (75 μl) using a pipette. Inoculated and control petioles were placed upright in sterilized moist chambers lined with sterilized filter paper, and incubated at 25 °C in the dark for 8 and 13 days.

For re-isolation of the inoculated fungi, five different samples were aseptically obtained (0.5 or 0.8 cm diameter) using a cork borer. Samples were taken at the inoculation site and at 1 and 2 cm above or below. Half of each sample was kept at −20 °C for light and scanning electron microscopy studies. The other half was surface-sterilized for 1 min in 1% sodium hypochlorite, washed three times with sterile distilled water and plated on potato dextrose agar (PDA, from Oxoid). The presence of the entomopathogenic fungi on the tissue samples was recorded after 8 days' incubation at 25 °C in the dark.

Other fungi present in palm tissues were also recorded. To study the presence of fungi naturally occurring in unwounded palm petioles four adult leaves were collected from Partida de Algorós (Elx, SE Spain). Petioles were cut, washed and surface-sterilized as described above. Fourteen samples from each petiole were aseptically obtained (0.8 cm diameter) using a cork borer. They were cut 1–1.5 cm from each other, surface-sterilized and plated as above.

2.1.2. Live palm petioles

Bioassays were carried out on living palms located at the Alicante University campus. Three to four petioles from 1–2 year-old (ca. 1 m high) palms were rubbed with 70% ethanol and 30 μl of conidial suspension was injected using syringe, but without razorblade wounding. *L.* c.f. *psalliotae* (isolated from the red scale insect *P. marlatti* in *P. dactylifera* leaves), *L. dimorphum* and *B. bassiana* isolate 33 were used. Inoculated areas were covered with Parafilm to avoid drying and external contamination. Fifteen and 30 days after inoculation, petioles were sampled and processed as described above, except that samples were also taken 3, 4 and 5 cm above and below the inoculation site.

Fragments were processed for microscopy and culture as described in Section 2.1.1. PDA used for plating out live palm tissue included 1000 μg ml^{-1} Triton X-100, 50 μg ml^{-1} of each Streptomycin, Penicillin and Rose Bengal B. The presence of the entomopathogenic fungi on the tissue samples was recorded up to 30 days after incubation at 25 °C in the dark. Live palm bioassays were performed twice (March–April 2004 and 2005).

2.2. Light (LM) and scanning electron microscopy (SEM)

Palm tissue samples, which showed growth of the entomopathogenic fungi on PDA plates, or uninoculated controls, were used for microscopy observations. For light microscopy frozen samples were embedded in OCT (Leica, Germany). The blocks were sectioned (20–50 μm thick) using a cryostat (Leica, CM 1510-1), and then stained with 0.1% (w/v) cotton blue in lactic acid. Samples from live palms were also stained with 0.01% (w/v) toluidine blue in 0.1 M KH_2PO_4–NaOH (pH 6) or 0.05% trypan blue in lactic acid for 45 min at 60 °C. Sections were viewed and photographed with an Olympus BHS light microscope or with a Leica DMLB microscope equipped with a Leica DFC480 camera. Alternatively, pieces of frozen samples were processed for SEM (Lopez-Llorca and Orts, 1994). They were fixed overnight at 4 °C in 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) and rinsed three times (10 min each) in phosphate buffer. Samples were then dehydrated in an ethanol series and critical point dried. The specimens were sputter-coated with gold and observed in a JEOL JSM 840 SEM at 10 kV or in a HITACHI S-3000 N SEM at 20 kV.

3. Results

3.1. Fungal colonization of date palm petioles

The wounds in the petiole samples showed hydropic and necrotic lesions at less than 1 cm above and below the wound in both control and inoculated petioles (Figs. 1–2).

3.1.1. Entomopathogenic fungi in detached petioles

Entomopathogenic fungi were isolated from inoculation sites (67–100%) from both young (except from one sampling occasion) and adult palms 8 or 13 days after incubation in sterile moist chambers (Table 1). *B. bassiana* 33 (in young palms) and *L. dimorphum* were both recovered 2 cm above and below the inoculation site, whereas *B. bassiana* 119 was mostly isolated at up to 1 cm from the inoculation site (Table 1). Entomopathogenic fungi were never isolated from non-inoculated petioles.

3.1.2. Entomopathogenic fungi in live palm petioles

The colonization of petiole fragments from live palms 15 and 30 days after inoculation on two consecutive years is shown in Table 2. All fungi were recovered from the inoculation site. *B. bassiana* colonized the petioles up to 3 cm above and below the inoculation site after 30 days in 2004, but less so the

Figs. 1–2. Live palm petioles showing hydropic and necrotic lesions. Control (1) and *Beauveria bassiana* (2) inoculated petioles; bars = 1 cm.

Table 1
Percentage of petiole fragments colonized by entomopathogenic (E) and other (O) fungi in detached palm petioles of two independent experiments

Fungus inoculated	Site[a]	Adult palms				Young palms				Adult palms				Young palms			
		8 days		13 days		8 days		13 days		8 days		13 days		8 days		13 days	
		E	O	E	O	E	O	E	O	E	O	E	O	E	O	E	O
Beauveria bassiana 119	A2	0	33	0	0	0	0	0	0	33	0	0	0	0	0	0	100
	A1	0	0	67	0	0	0	0	67	0	0	33	0	33	33	33	67
	INS	100	33	67	67	100	33	0	100	100	33	100	67	100	33	67	67
	B1	33	0	33	33	0	0	33	33	33	0	33	0	67	0	33	100
	B2	0	0	0	33	0	67	0	67	0	0	0	0	0	0	0	100
Lecanicillium dimorphum	A2	0	0	33	0	33	33	33	33	0	33	0	0	0	0	33	33
	A1	33	0	0	0	33	33	33	67	33	0	33	0	0	0	67	33
	INS	67	0	67	67	67	67	100	67	100	0	100	67	100	0	100	100
	B1	33	0	0	33	33	33	33	33	0	33	0	33	33	0	33	33
	B2	0	33	0	33	0	0	67	33	33	33	33	67	67	0	33	67
Beauveria bassiana 33	A2	n.d.	n.d.	n.d.	n.d.	33	67	0	67	n.d.	n.d.	n.d.	n.d.	67	0	0	0
	A1	n.d.	n.d.	n.d.	n.d.	100	33	33	33	n.d.	n.d.	n.d.	n.d.	100	0	67	0
	INS	n.d.	n.d.	n.d.	n.d.	100	0	67	33	n.d.	n.d.	n.d.	n.d.	100	0	100	33
	B1	n.d.	n.d.	n.d.	n.d.	67	33	67	67	n.d.	n.d.	n.d.	n.d.	67	67	67	0
	B2	n.d.	n.d.	n.d.	n.d.	33	33	0	0	n.d.	n.d.	n.d.	n.d.	33	0	33	0
Control (none)	A2	0	33	0	0	0	33	0	33	0	0	0	n.d.	0	0	0	33
	A1	0	0	0	0	0	67	0	33	0	0	0	n.d.	0	0	0	33
	INS	0	33	0	0	0	67	0	33	0	33	0	n.d.	0	33	0	33
	B1	0	0	0	33	0	67	0	33	0	0	0	n.d.	0	0	0	0
	B2	0	33	0	0	0	33	0	0	0	67	0	n.d.	0	0	0	67

($n = 3$); n.d., Not determined.
[a] INS: site of injection; A1, A2 and B1, B2: site 1 or 2 cm above (A) or below (B) site of injection.

Table 2
Percentage of petiole fragments colonized by entomopathogenic (E) and other (O) fungi in live palm petioles experiments ($n = 3–4$)

Fungus inoculated	Site[a]	2004				2005			
		15 days		30 days		15 days		30 days	
		E	O	E	O	E	O	E	O
Beauveria bassiana 33	A3	0	100	33	33	0	0	0	50
	A2	0	100	100	33	25	50	0	25
	A1	67	0	67	100	25	50	0	25
	INS	67	100	33	100	50	75	75	50
	B1	33	33	33	67	25	50	0	25
	B2	33	67	0	67	25	50	0	25
	B3	0	33	67	67	0	0	25	100
Lecanicillium dimorphum	A3	0	0	0	67	0	75	0	0
	A2	0	33	0	67	0	25	0	33
	A1	0	0	0	67	25	25	33	33
	INS	33	67	100	100	50	75	67	67
	B1	33	33	0	67	25	100	33	33
	B2	0	0	0	100	0	50	0	33
	B3	0	0	0	67	0	50	0	0
Lecanicillium c.f. *psalliotae*	A3	0	67	0	33	25	0	25	0
	A2	0	0	0	33	25	0	0	50
	A1	0	67	0	33	50	50	25	25
	INS	100	100	100	100	100	75	50	75
	B1	0	33	33	67	50	25	0	0
	B2	33	0	33	33	25	0	0	0
	B3	0	0	0	33	0	25	0	0
Control (none)	A3	0	33	0	33	0	50	0	75
	A2	0	67	0	67	0	0	0	50
	A1	0	67	0	33	0	25	0	100
	INS	0	100	0	100	0	100	0	100
	B1	0	67	0	33	0	50	0	50
	B2	0	100	0	100	0	100	0	25
	B3	0	67	0	67	0	0	0	100

[a]INS: site of injection; A1–A3 and B1–B3: site 1–3 cm above or below site of injection.

following year. *L. dimorphum* colonized the petioles to a lesser extent than *B. bassiana* and was never detected further than 1 cm from the inoculation site. *L.* c.f. *psalliotae*, which was excluded in the detached petiole study, colonized the leaf petioles up to 3 cm above and 2 cm below the inoculation site after 15 days (2005). There was no colonization at 4–5 cm above or below the inoculation site by any of the three fungi studied (not shown). Entomopathogenic fungi were never isolated from non-inoculated petioles.

3.1.3. Non-entomopathogenic fungi isolated from date palm petioles

Contaminating fungi were isolated from both detached (Table 1) and live (Table 2) petioles, but to a lesser extent from the former. Results from the experiments with live petioles are summarized in Table 3. In the unwounded petioles sterile mycelia dominated, while *Alternaria alternata* was found in all wounded treatments. In all treatments *Cladosporium cladosporoides* and Coelomycetes including *Phoma* spp. were also detected. Other fungi were occasionally isolated, but usually with low incidence rates (1–3%). Bacteria (in ca. 18% of all petioles tested) and yeasts (ca. 1%) were also found. All petiole fragments used for the microscopy studies were selected free from contaminating organisms.

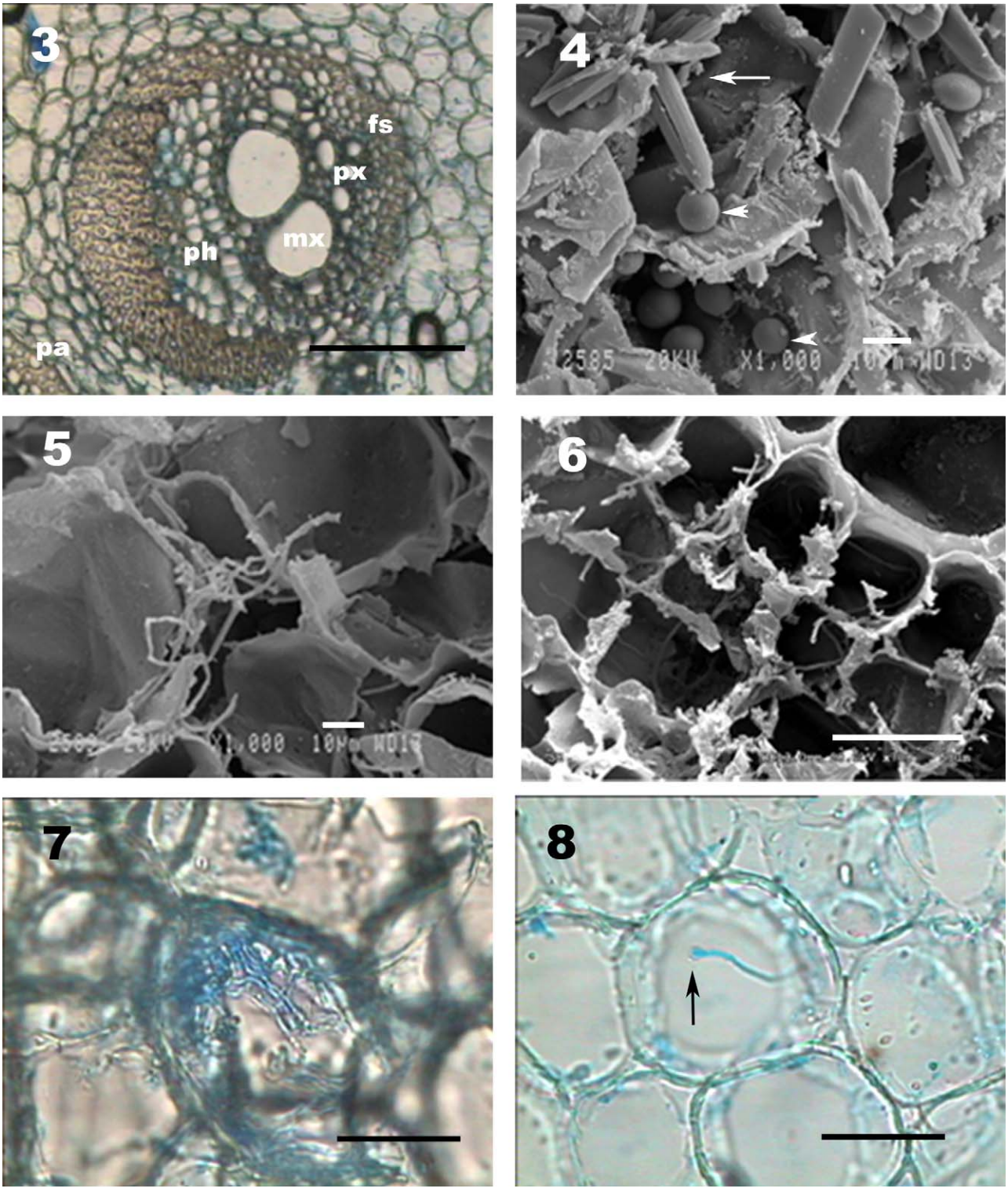
3.2. Light (LM) and scanning electron microscopy (SEM)

3.2.1. Detached petioles

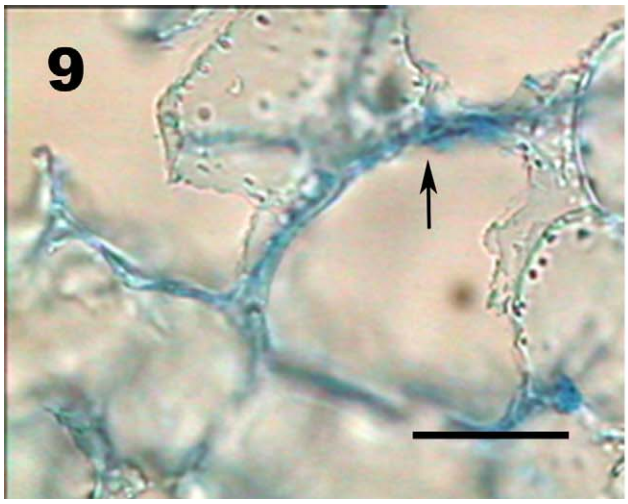
Uninoculated petioles showed no fungal colonization. The petiole sections showed the typical monocot tissue anatomy

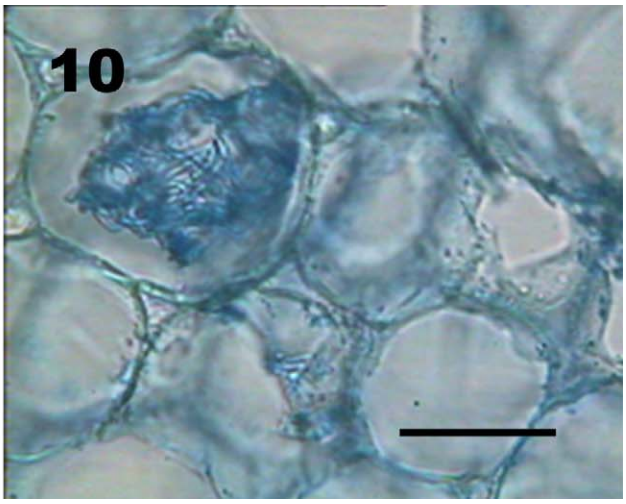
Table 3
Isolation of non-entomopathogenic fungi during experiments using live palm petiols

Treatment	*n*	Percentage isolations	Major genera
Unwounded control	56	30	Sterile mycelia (13%)
Wounded control	154	59	*Alternaria* (36%), *Cladosporium* (11)
Beauveria bassiana 33	154	48	*Alternaria* (25%)
Lecanicillium dimorphum	143	47	*Alternaria* (22%), Coelomycetes (11%), *Cladosporium* (10%)
Lecanicillium c.f. *psalliotae*	154	34	*Alternaria* (18%)



Figs. 3–8. Detached palm petioles colonized by *Beauveria bassiana* and uninoculated controls. (3) LM showing transverse section of parenchymatic (pa) of uninoculated date palm petiole and vascular bundle: metaxylem (mx), protoxylem (px), phloem (ph), fibrous bundle sheath (fs) of uninoculated date palm petiole tissue; bar = 12 μm. (4) SEM of uninoculated date palm petiole tissue. Starch grains (arrow heads) and calcium oxalate crystals (arrow) in parenchymatic cells; bar = 10 μm. (5) SEM of *B. bassiana* in petiole tissue inoculated and incubated for 8 days showing hyphae in intercellular spaces of parenchymatic cells; bar = 10 μm. (6) SEM of *B. bassiana* inoculated and incubated for 13 days showing mycelium in parenchyma intercellular spaces and associated with parenchymatic cells; bar = 50 μm. (7) LM showing a parenchymatic cell associated with blue-stained mycelium; bar = 6 μm. (8) LM showing a penetration structure, appressoria (arrow), of *B. bassiana* in parenchymatic cell observed by LM; bar = 6 μm.





Figs. 9–10. Detached petioles colonized by *Lecanicillium dimorphum*. (9) LM of *L. dimorphum* in petiole tissue inoculated and incubated for 8 days. Hyphae (stained blue, arrow) in intercellular spaces of parenchyma; bar = 45 μm. (10) LM of *L. dimorphum* in petiole tissue inoculated and incubated for 13 days showing mycelum inside a parenchyma cell; bar = 45 μm.

with parenchyma and vascular tissue particularly apparent (Fig. 3). Parenchymatic cells often showed starch grains (Fig. 4, arrowheads) and calcium oxalate crystals (arrow).

B. bassiana samples incubated for 8 days mainly colonized parenchyma, especially in the intercellular spaces (Fig. 5). *B. bassiana* samples incubated for 13 days showed a higher hyphal density than the 8 day samples, where the fungus colonized parenchyma both intra- and intercellularly (Fig. 6). *B. bassiana* petiole samples showed abundant mycelial growth within parenchymatic cells (Fig. 7) and vascular tissues (not shown). Apical swellings of the hyphae resembling appressoria were occasionally found (Fig. 8).

L. dimorphum samples inoculated and incubated for 8 days showed colonization of petiole parenchyma, mainly growing in the intercellular spaces (Fig. 9, arrows). *L. dimorphum* petiole samples incubated for 13 days mainly showed parenchyma colonization (Fig. 10).

3.2.2. Live palm petioles

Control petioles showed no fungal growth and contained abundant starch granules within parenchyma cells (Fig. 11). Fifteen days after inoculation *B. bassiana* extensively colonized parenchyma cells inter- and intracellularly (Fig. 12). The fungus was also found in vascular (protoxylem) tissue (Fig. 13).

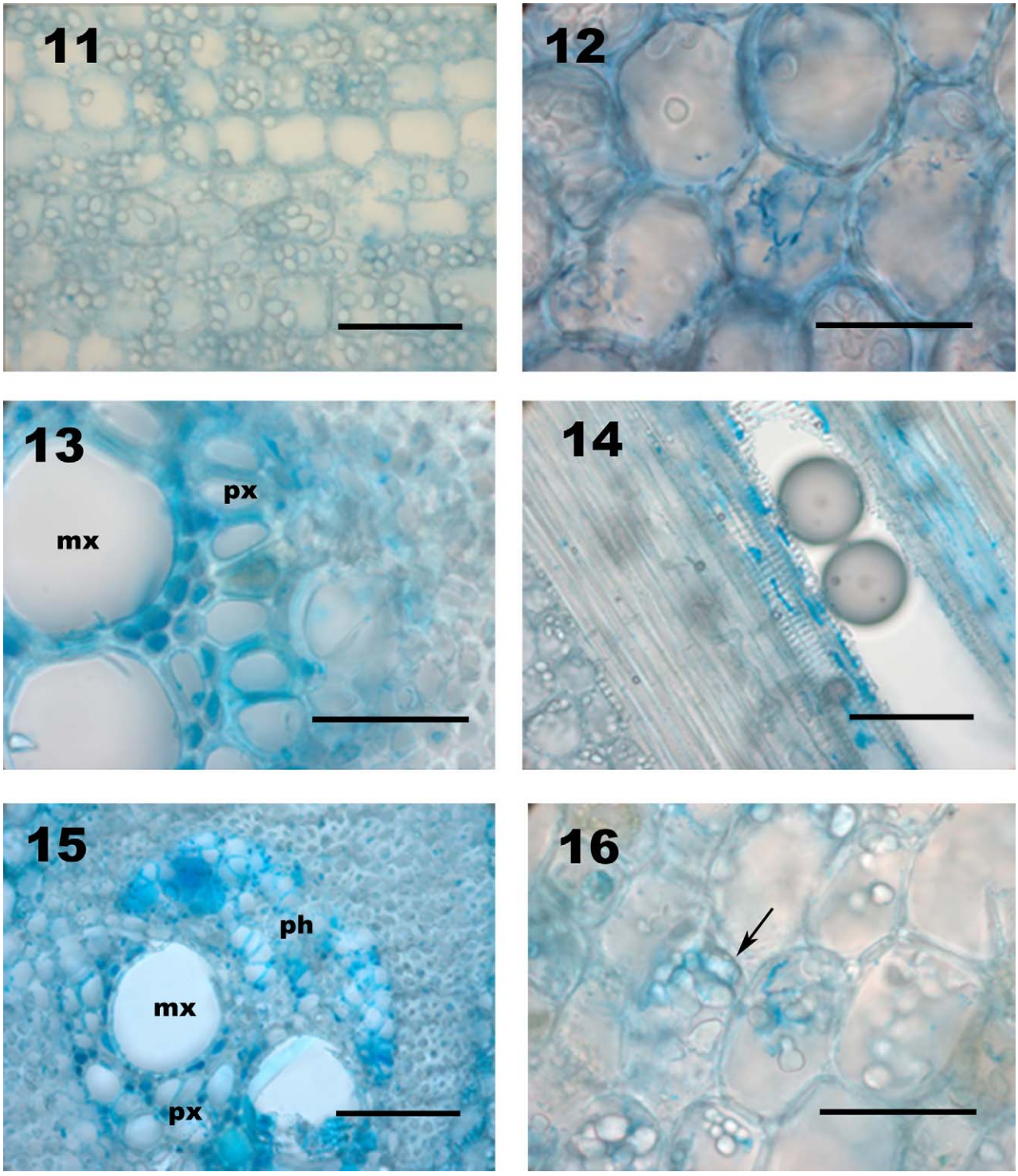
L. dimorphum was located in vascular tissue both 15 and 30 days after inoculation (Fig. 14). Colonization was particularly obvious in transverse sections (Fig. 15), including phloem vessels. Parenchyma cells were also colonized (Fig. 16). Starch granules appeared to be colonized by fungal hyphae (Fig. 16, arrow).

L. c.f. *psalliotae* grew in the vascular tissues (Fig. 17), and was also present in parenchyma cells apparently colonizing starch granules (Fig. 18).

4. Discussion

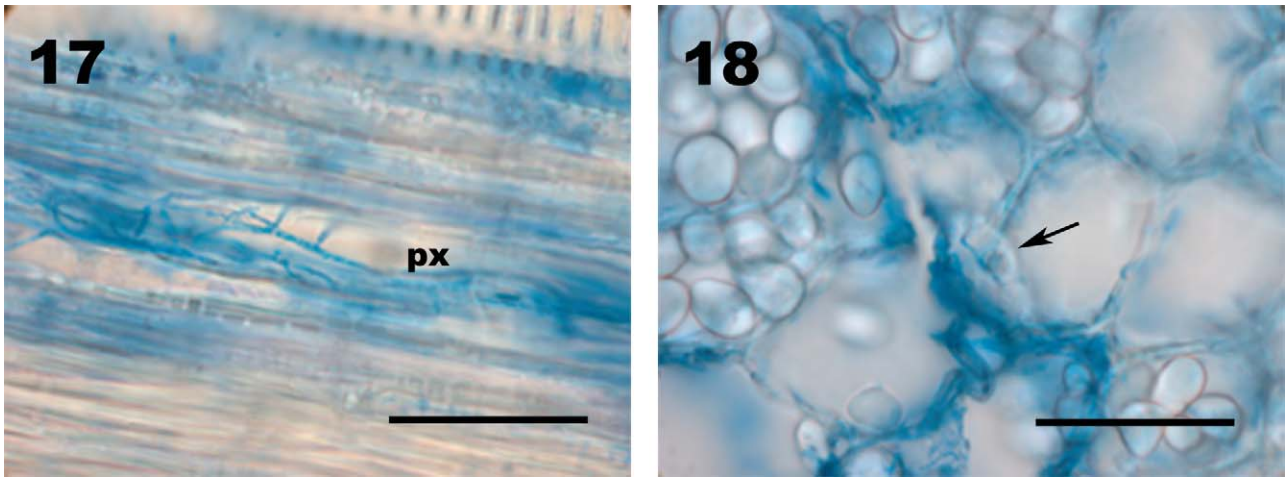
The entomopathogenic fungi were able to colonize date palm tissues after petiole wounding. *B. bassiana* (isolates 119 and 33) and *L. dimorphum* were always recovered from the injection sites from both young and adult palms after 13 days incubation in moist chambers. These fungi were isolated at least 2 cm from the inoculation site. The results confirmed that entomopathogenic fungi, applied in the laboratory, survived endophytically and colonized petiole tissue of young and adult date palms. In field studies, the three entomopathogenic fungi used for inoculation (*B. bassiana* 33, *L. dimorphum* and *L.* c.f. *psalliotae*) were detected in live young palms at the inoculation site after 15 and 30 days in the two years of study. There was colonization 3 cm above or below the inoculation site for *B. bassiana* and 3 cm above the inoculation site for *L.* c.f. *psalliotae*, whereas *L. dimorphum* was never detected further than 1 cm from the inoculation site. In only two instances, *L. dimorphum* in detached petioles and *L.* c.f. *psalliotae* in live petioles, there appeared to be a ‘‘gap’’ in the logical order of petiole colonization by the entomopathogenic fungi. This could be an artefact due to the inoculation technique (injection of living petiols) or to lack of vascular flow (for detached petiols), *B. bassiana* applied to whorl-stage corn by foliar application or injection colonized and moved within the plants, and persisted to provide season-long suppression of the European corn borer *O. nubilalis* (Bing and Lewis, 1991). *B. bassiana* was isolated from the node above the injection site. *B. bassiana* movement within corn could be attributed to either passive transport within the xylem and/or to mycelial growth (Bing and Lewis, 1991; Wagner and Lewis, 2000). We could explain the movement of entomopathogenic fungi within date palm petioles by these mechanisms. Although *B. bassiana* is a ubiquitous entomopathogenic fungus in soil, certain isolates have been found colonizing plant tissue (Bing and Lewis, 1993). In uninoculated petiole fragments or leaves we did not isolate any of the entomopathogenic fungi used in our experiments.

Inoculation of date palm petioles with the entomopathogenic fungi did not show disease symptoms as compared to non-inoculated wound controls. The necrotic and hydropic zones were never longer than 1 cm after incubation for 30 days. In



Figs. 11–16. Live palm petioles colonized by *Beauveria bassiana*, *Lecanicillium dimorphum* and uninoculated control. (11) LM of parenchyma of uninoculated control showing abundant starch grains; bar = 100 μm. (12) LM of *B. bassiana* in parenchyma 15 days after inoculation; bar = 50 μm. (13) LM of *B. bassiana* in vascular tissue 15 days after inoculation: metaxylem (mx), protoxylem (px); bar = 50 μm. (14) LM showing colonization by *L. dimorphum* of vascular tissues 15 days after inoculation; bar = 100 μm. (15) LM showing colonization by *L. dimorphum* of vascular tissue 30 days after inoculation: metaxylem (mx), protoxylem (px), phloem (ph); bar = 100 μm. (16) LM showing *L. dimorphum* colonizing parenchyma and associated with starch granules (arrow) 30 days after inoculation; bar = 50 μm.

contrast, the phytopathogenic fungus *Penicillium vermoesenii* showed 5–7 cm long necrosis after incubation for 25 days on *Washingtonia filifera* (Lopez-Llorca and Orts, 1994). *P. vermoesenii* also developed 15 cm long hydropic symptoms on date palm petioles 25 days after inoculation (F.J. Fernández Navarro, unpublished). It appears that entomopathogenic fungi cause few symptoms on the inoculated petioles and we therefore regard these fungi as endophytic colonizers of date palm tissues.



Figs. 17–18. Live palm petioles colonized by *Lecanicillium* c.f. *psalliotae.* (17) LM showing colonization of protoxylem (px) by *L.* c.f. *psalliotae* 15 days after inoculation; bar = 50 μm. (18) LM showing colonization by *L.* c.f. *psalliotae* in palm tissue and associated with starch granules (arrow) 15 days after inoculation; bar = 50 μm.

The technique used to inoculate the petioles (by wounding and injection) directly on the palms and covering the wound with Parafilm obviously did not protect the inoculation site from contamination by other microorganisms, especially fungi such as *A. alternata* and *C. cladosporioides*. *C. cladosporioides* and various Coelomycetes were found in all treatments, but not all fragments, (including non-wounded controls) and indicate that these fungi may be natural endophytes of date palms.

Colonization of detached petioles by entomopathogenic fungi cannot be considered proof of their endophytism. This approach however gave us an insight on the behaviour of these antagonists within palm tissue. In a previous study, dry tissue of several palm species was found to be colonized and used as a substrate for growth and sporulation by several antagonistic fungi, including entomopathogens (Lopez-Llorca et al., 1999).

In histological sections, uninoculated *P. dactylifera* petioles showed the typical monocot tissue anatomy with parenchyma and vascular tissue particularly apparent. Crystals were sometimes found on the surface of the plant tissue sample observed. Similar crystals were also found in tissue of other *Phoenix* spp. (Lopez-Llorca et al., 1999) and in *W. filifera* (Lopez-Llorca and Orts, 1994). They are probably made of calcium oxalate (Tomlinson, 1990) and formed by plant organic acid and salt crystallization (Raven et al., 1992; Paniagua et al., 1996).

In our study, inter- and intracellular growth of entomopathogens (*B. bassiana* and *L. dimorphum*) in parenchyma was particularly abundant. This resembles the behaviour of *P. vermoesenii*, a wound pathogen of palms (Lopez-Llorca and Orts, 1994). Appressoria were detected on hyphae of *B. bassiana* and *L. dimorphum* growing intracellularly in detached petioles. This fungus was found to penetrate the cuticle of dried palm leaves with and without these structures (Lopez-Llorca et al., 1999). Resources for colonization of parenchyma by entomopathogenic fungi were possibly obtained by degradation of starch. Typical starch granules particularly abundant in young palm parenchyma cells were often colonized by hyphae.

Entomopathogenic fungi were also found in vascular vessels. This fact could explain fungal colonization by means of vascular bundle interconnections (Paniagua et al., 1996; Wagner and Lewis, 2000). We did not find any evidence of fungi completing their anamorphic cycle in date palm petioles. We have, however, found extensive conidial production by entomopathogens on the surface of dried palm leaves (Lopez-Llorca et al., 1999).

In conclusion, we provide evidence that important entomopathogenic fungi colonized living palm tissue and therefore may act as palm endophytes. This behaviour may be a suitable tool for designing biocontrol strategies targeted at palm pests and diseases (Asensio et al., 2005). In our study, however, we have encountered several complications. Non-axenic tissue of plants grown in the field, although representing the real target, also contains contaminating fungi. Some of these may be true endophytes, whereas others are common phylloplane dwellers. The latter invade the palm tissue through the wounds used for inoculating the entomopathogens. Better inoculation and tracing techniques by means of specific molecular markers should be used in future studies. Our laboratory has earlier provided evidence on the induction of plant defences by other biocontrol fungi (Bordallo et al., 2002). Studies on the proteome of palms inoculated with entomopathogens aimed at exploring the induction of PR-related proteins are presently in progress (Gómez-Vidal et al., unpublished).

Acknowledgements

This work was financed by the Spanish Ministerio de Ciencia y Tecnología with a P4 Project (AGL2000-0342-P4-02), and a scholarship from the Spanish Ministry for Education and Science to SGV (AP2001-1387). We thank Viveros Huerto del Cura S.A. for technical support.

References

Asensio, L., Lopez-Llorca, L.V., López-Jiménez, J.A., 2005. Use of light, scanning electron microscopy and bioassays to evaluate parasitism by

entomopathogenic fungi of the red scale insect of palms (*Phoenicococcus marlatti* Ckll., 1899). Micron 36, 169–175.

Azevedo, J.L., Maccheroni, W., Pereira, J.O., Araujo, W.L., 2000. Endophytic microorganisms: a review on insect control and recent advances on tropical plants. Electron. J. Biotechnol. 3, 40–65.

Bing, L.A., Lewis, L.C., 1991. Suppression of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) by endophytic *Beauveria bassiana* (Balsamo) Vuillemin. Environ. Entomol. 20, 1207–1211.

Bing, L.A., Lewis, L.C., 1992a. Endophytic *Beauveria bassiana* (Balsamo) Vuillemin in corn: the influence of the plant growth stage and *Ostrinia nubilalis* (Hübner). Biocontrol Sci. Technol. 2, 39–47.

Bing, L.A., Lewis, L.C., 1992b. Temporal relationships between *Zea mays*, *Ostrinia nubilalis* (Lep.: Pyralidae) and endophytic *Beauveria bassiana*. Entomophaga 37, 525–536.

Bing, L.A., Lewis, L.C., 1993. Occurrence of the entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin in different tillage regimes and in *Zea mays* L. and virulence towards *Ostrinia nubilalis* (Hübner) Agriculture Ecosyst. Environ. 45, 147–156.

Bordallo, J.J., Lopez-Llorca, L.V., Jansson, H.-B., Salinas, J., Persmark, L., Asensio, L., 2002. Colonization of plant roots by egg-parasitic and nematode-trapping fungi. New Phytol. 154, 491–499.

Bultman, T.L., Bell, G., Martin, W.D., 2004. A fungal endophyte mediates reversal of wound-induced resistance and constrains tolerance in a grass. Ecology 85, 679–685.

Bultman, T.L., Conard, N.J., 1998. Effects of endophytic fungus, nutrient level, and plant damage on performance of fall armyworm (Lepidoptera: Noctuidae). Environ. Entomol. 27, 631–635.

Lodge, D.J., Fisher, P.J., Sutton, B.C., 1996. Endophytic fungi of *Manilkara bidentata* leaves in Puerto Rico. Mycologia 88, 733–738.

Lopez-Llorca, L.V., Carbonell, T., Salinas, J., 1999. Colonization of plant waste substrates by entomopathogenic and mycoparasitic fungi - a SEM study. Micron 30, 325–333.

Lopez-Llorca, L.V., Orts, S., 1994. Histopathology of infection of the palm *Washingtonia filifera* with the pink bud rot fungus *Penicillium vermosenii*. Mycol. Res. 98, 1195–1199.

Paniagua, R., Nistal, M., Sesma, P., Álvarez-Uría, M., Fraile, B., 1996. Citología e histología vegetal y animal. Biología de las células y tejidos animales y vegetales. Interamericana-McGraw-Hill, Madrid.

Raven, P.H., Evert, R.F., Eichhorn, S.E., 1992. Biology of Plants, fifth ed. Worth Publishers, New York.

Schulz, B., Boyle, C., Draeger, S., Römmert, A., Krohn, K., 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. Mycol. Res. 106, 996–1004.

Tomlinson, P.B., 1990. The Structural Biology of Palms. Oxford University Press, Oxford, England.

Wagner, B.L., Lewis, L.C., 2000. Colonization of corn, *Zea mays*, by the entomopathogenic fungus *Beauveria bassiana*. Appl. Environ. Microbiol. 66, 3468–3473.