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Endophytic colonisation of opium poppy, *Papaver somniferum*, by an entomopathogenic *Beauveria bassiana* strain

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Abstract

Beauveria bassiana strain EABb 04/01-Tip isolated from stem-borer larvae of *Timaspis papaveris* (Hymenoptera: Cynipidae), a serious pest of opium poppy in Spain, was shown to be able to become established endophytically in this pharmaceutical crop. Microbiological, molecular and light and electron microscopic methods were used to study fungal colonisation and to describe its mode of penetration. After inoculation with a foliar spray of conidia, microbiological methods showed 100% of plants examined 24, 48, 72 and 144 h after treatment to be colonised endophytically by the fungus, although the percentage of previously surface sterilised leaf pieces showing fungal growth was 100% at 24 and 48 h, and 80 and 75% at 72 and 144 h after treatment, respectively. The fungus was also observed in leaf pieces obtained from newly formed leaves, indicating that it could spread from treated leaves to leaves formed after fungal application. For molecular studies, a polymerase chain reaction (PCR) protocol was used to amplify the ITS1-5.8S-ITS2 regions of the rDNA of the plant and the fungus. This procedure allowed the detection of the fungus on the surface of the leaves and also endophytically, but only at 72 h after treatment. A nucleotide BLAST search revealed that the ITS1-5.8S-ITS2 sequence of strain EABb 04/01-Tip showed 100% homology with a similar sequence from *Cordyceps bassiana*. SEM images revealed that although numerous conidia were observed on the leaf surface, few germinated and penetrated. Intracellular colonisation by *B. bassiana* was not observed, but hyphae were detected growing into the xylem vessels. The fungus was found to colonise 40.5 ± 4.3% of seedlings (with two cotyledons and the two first real leaves) from seeds dressed with a fungal spore suspension. These results may have implications in the biological control of *T. papaveris*, including the possible systemic protection of the plant against this cynipid.

Key words: biological control, endophyte, entomopathogenic fungi, *Papaver somniferum* cv. *nigrum*, stem-borer, systemic action, systemic protection, *Timaspis papaveris*

Introduction

The opium poppy, *Papaver somniferum* L., is an economically important pharmaceutical crop in Spain. About 7400 ha are cultivated annually producing a yield averaging over 4800 tonnes (straw) [1] that are used as a source for codeine and morphine. Whilst there are some insect species that may feed on this crop, the weevil *Ceutorrhynchus macula-alba* Herbst. (Coleoptera: Curculionidae) and *Timaspis papaveris* (Kieffer) (Hymenoptera: Cynipidae) are the main insect pests of opium poppy in Spain [2–4]. Females of *T. papaveris* lay their eggs in the stem, and after hatching larvae burrow and feed inside the stem, thereby causing important yield losses. Chemical control of *T. papaveris* is difficult because egg, larval and pupal stages are endophytic, and alternative

control methods are, therefore, urgently needed. Recently, a strain of *Beauveria bassiana* (teleomorph *Cordyceps bassiana*) was found infecting *T. papaveris* larvae within stems of opium poppy plants. This strain might provide systemic protection against damage by *T. papaveris* in opium poppy if it had endophytic capacity. Previous studies have shown that *B. bassiana* forms an endophytic symbiosis with maize providing suppression of European corn borer *Ostrinia nubilalis* (Hübner) [5, 6] and *Sesamia calamistis* Hampson [7]. In the present study, we used microbiological, molecular and electron microscopic techniques to elucidate whether our *B. bassiana* strain could penetrate and colonise opium poppy.

Materials and methods

Fungal strain and cultivation

The *B. bassiana* strain EABb 04/01-Tip (C.R.A.F. University of Cordoba Entomopathogenic Fungi Collection, Cordoba, Spain) was isolated from dead *T. papaveris* larvae from a field in Carmona (Seville) (Figure 1a). Monosporic cultures of this strain were grown on slants of Malt Agar (MA) at 25 °C in the dark and stored at 4 °C. To obtain a spore suspension, the isolate was grown on Petri plates on MA for 15 days at 25 °C in the dark. Petri plates were sealed with parafilm, and freshly collected conidia from 15-day-old cultures were used in the experiments. Conidial suspensions were prepared by scraping conidia from Petri plates into an aqueous sterile solution of 0.002% Tween 80. The conidial suspensions were filtered through several layers of cheesecloth to remove mycelium mats. To homogenise the inoculum, the conidial suspensions were sonicated for 10 min (P-selecta ultrasons). Concentrations of viable conidia used for inoculation were calculated using the colony forming unit's method [8].

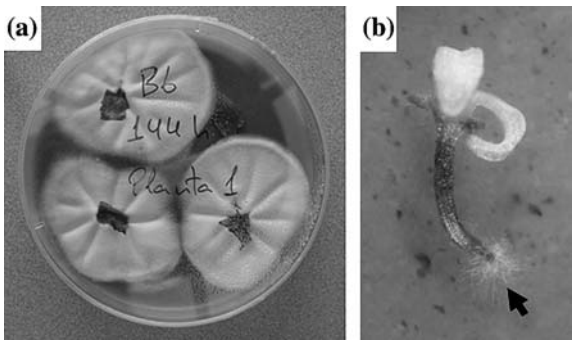

Figure 1. (a) Petri plate with three leaf pieces of opium poppy sprayed 144 h before with a fungal suspension of 7.5×10^8 spores/ml showing growth of *Beauveria bassiana* strain EABb 04/01-Tip. (b) Opium poppy seedling from seeds dressed with 5.5×10^8 spores/ml of *B. bassiana* strain EABb 04/01-Tip showing polar growth of the fungus (arrow).

Plants

Opium poppy plants, *Papaver somniferum* cv *nigrum*, were grown in environmental chambers at 21 °C, 60–90% relative humidity, and a 12-h photoperiod (fluorescent light; 360 μE m^{-2} s^{-1}). In all experiments, treatments (plants in pots or plants in Petri dishes) were arranged in a randomised complete block design.

Inoculation of opium poppy plants

Colonisation of plants by *B. bassiana* was determined 24, 48, 72 and 144 h after treatment. Five spore suspensions were separately obtained from different slant cultures of isolate 04/01-Tip and each suspension was adjusted to 7.5×10^8 spores/ml. Five-week-old poppy plants having 4–5 leaves were sprayed with the conidial suspensions of *B. bassiana* or with an aqueous sterile solution of 0.002% Tween 80 (controls). Series of four plants were randomly selected and treated with each of the five-conidial suspensions or with water using one plant from each series for each sampling time. Thereby, there were five replicates (plants) per time interval and treatment. Plants were watered by spraying them with sterile water as needed.

Determination of endophytic colonisation by microbiological, molecular and electron microscopic techniques

At 24, 48, 72 and 144 h after treatment, the endophytic colonisation was ascertained by re-isolation following surface sterilisation of 2 mm^2 (1×2 mm) segment of each of the leaves included in a sample (five plants per time interval for *B. bassiana* treatment and for controls). The sections were firstly cut and then surface-sterilised with 1% sodium hypochlorite for 3 min, rinsed twice in sterile distilled water and then placed on

sterile wet filter paper. Using sterile techniques, the above leaf pieces were placed on *B. bassiana* selective medium [8] (2% oatmeal infusion, 2% agar, 550 μg/ml dodine, 5 μg/ml chlortetracycline and 10 μg/ml crystal violet). The presence or absence of *B. bassiana* growth on the leaf samples was recorded after 10 days of incubation at 25 °C. Plants tested for endophytic colonisation 144 h after treatment had 1–2 newly formed leaves that were also included in our study. The percentage of leaves showing positive isolation at each sampling interval was compared statistically using a Tukey (HSD) test ($P = 0.05$).

For molecular studies, a polymerase chain reaction (PCR) protocol was used to amplify the ITS1-5.8S-ITS2 regions of the rDNA using the universal pair of primers ITS1/ITS4 and the PCR conditions described before [9]. Leaf segments (10×10 mm) taken randomly from fungal or control treatments of the same plants as above before or after surface sterilisation were used. Positive PCR controls included a conidial suspension of *B. bassiana* and poppy seeds and seedlings germinated and grown under sterile conditions as described below. For DNA extraction, leaf segments cut in small pieces with a sterile blade, or 500 μl of the conidial suspension were placed in a 1.5-ml Fast DNA tube containing lysing matrix A and 500 μl of CLS-VF solution and 200 μl of Protein Precipitation Solution (PPS) (for plant material), or CLS-Y solution (for conidial suspension). Cells were mechanically disrupted in a Fast Prep System Bio 101 (Qbiogene, Madrid, Spain) and processed with the Fast DNA kit according to the manufacturer's instructions. After amplification, PCR products were separated on 1.5% agarose gels in 1× TAE for 2 h at 100 V, visualised by ethidium bromide staining, and scored by comparison to a 0.1-kb DNA ladder XIV size marker (Roche Diagnostics, Mannheim, Germany). Additionally, the ITS1-5.8S-ITS2 regions of the rDNA from *B. bassiana* or opium poppy were purified with a gel extraction kit (GENECLEAN Turbo; Qbiogene, Madrid, Spain) and used for direct DNA sequencing by using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Madrid, Spain) according to manufacturer's instructions. The ITS1-5.8S-ITS2 sequences for *B. bassiana* and for *P. somniferum* cv *nigrum* are deposited in the GenBank nucleotide sequence database with Accession Numbers DQ364698 and DQ364699, respectively.

For light and transmission electron microscopy studies, we used the same plants, and cut leaves into small pieces (1× 2 mm) that were fixed overnight at 4 °C with 0.25% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. After three rinses with sodium cacodylate buffer, the fragments were post-fixed for 90 min at 4 °C in 0.2% osmium tetroxide and dehydrated in a series of acetone baths from 40 to 100%. After infiltration overnight with an epon/acetone mixture (1/1 v) they were embedded in Epon 812. Ultrathin sections (5 nm–3 μm) cut with a glass knife on an LKB NOVA ultramicrotome were stained with 1% toluidine blue (light microscopy study) and observed with a Leica DM6000 B photomicroscope or with uranyl acetate and lead citrate (electron microscopy study) and examined using a Philips CM-10 electron microscope operated at 80 kV.

Leaf pieces for conventional SEM were fixed according to the preceding procedure and then dehydrated in an ethanol series to 100% ethanol. Leaves were critical point dried in a drying unit with CO_2 (Balzers CPD-030). Leaf pieces were coated with gold–palladium (20:80) in a Polaron E5100 sputter coating unit and viewed on a JEOL JSM 6300 SEM at 39 kV. In order to make an estimate of the number of conidia actually germinating, we obtained several SEM images from the leaf surface, 50 conidia each, and we measured the number of germinating and penetrating conidia.

Seed dressing with Beauveria bassiana

Opium poppy seeds were surface-sterilised with 10% sodium hypochlorite for 3 min, rinsed twice in sterile distilled water and they were coated to runoff with a spore suspension of 5.5×10^8 spores/ml that was sprayed under agitating conditions. Finally, they were placed on sterile wet filter paper in sterile Petri dishes. Control seeds were treated similarly with 0.002% Tween 80. Seeds were plated for germination on four Petri dishes, 20 seeds each, with a piece of wet filter paper in humid conditions (100% RH) in the above environmental chambers to stimulate germination. After germination, the seeds were left in the sterile Petri plates. Ten small seedlings randomly selected from each replicate with two cotyledons and the two first real leaves

were surface sterilised with 1% sodium hypochlorite for 3 min, rinsed twice in sterile distilled water, and placed on *B. bassiana* selective medium [8]. The presence or absence of *B. bassiana* growth on the seedlings was recorded as above.

Results and discussion

B. bassiana was isolated from all opium poppy plants that were sprayed with the fungal suspension of 7.5×10^8 spores/ml. The percentage of leaf pieces showing fungal growth when placed on *B. bassiana* selective medium was 100% at 24 and 48 h, and 80 ± 4.5% and 75 ± 3.5% at 72 and 144 h after treatment, respectively (Figure 1a). In this latter interval, 144 h, although the percentage of isolation was significantly (Tukey test; $P < 0.05$) smaller than the previous sampling intervals, we detected the growth of the fungus in leaf pieces obtained from newly formed leaves, indicating that the fungus could spread from treated leaves to leaves formed after fungal application. Leaf pieces from controls did not exhibit any sign of *B. bassiana* growth when placed on *B. bassiana* selective medium. After seed dressing with *B. bassiana*, we also observed endophytic colonisation of cotyledons or seedlings (Figure 1b). The fungus was recovered from leaves of 40.5 ± 4.3% of seedlings from treated seeds while it was not found in any of the controls. The only previous demonstration of endophytic colonisation following foliar applications of *B. bassiana* was that in corn [6]. However, from a previous review on *B. bassiana* as a dual-purpose biocontrol organism [10], we know that it has also been found inside the tissues of potato, cotton, jimsonweed (*Datura stramonium*), and common cocklebur (*Xanthium strumarium*) [11]. A possible endophytic colonisation of coffee by *B. bassiana* has also been suggested [12], and endophytic colonisation of banana by weakly pathogenic *Fusarium* sp. and *Paecilomyces* sp. has been noted [13, 14]. Our work demonstrated for the first time endophytic colonisation of opium poppy by *B. bassiana* following either foliar applications or seed dressing.

PCR amplifications of total genomic DNA obtained from a conidial suspension of *B. bassiana* produced a single PCR product of about 570 bp (Figure 2, lane 1). A larger single PCR product of about 750 bp (Figure 2, lanes 2 and 3) was generated from total genomic DNA obtained from surface sterilised opium poppy seeds or seedlings grown under sterile conditions. The bands from lanes 1 and 2 were excised and sequenced. A nucleotide BLAST search [15] revealed that the *B. bassiana* and opium poppy sequences share 100% similarity with two reported ITS1-5.8S-ITS2 sequences from *C. bassiana* and *P. somniferum* (Genbank Accesion numbers AB079126 and AY689330, respectively). Two bands each corresponding in mobility to the bands from either *B. bassiana* or opium poppy, were found for leaves at 24 and 48 h after treatment with *B. bassiana* conidial suspension and processed for DNA extraction before the surface sterilisation procedure (Figure 2, lines 5 and 8) indicating the presence of fungal inoculum over the leaf surface. This band was not detected after 72 or 144 h (Figure 2, lines 12, 14 and 15) probably due to inoculum being washed-off when plants were sprayed with water. A band corresponding to *B. bassiana* was only detected for leaves surfaced sterilised and treated with *B. bassiana* conidial suspension at 72 h after the treatment (Figure 2, line 11). *B. bassiana* bands from lines 5, 8 and 11 were sequenced and found to have 100% sequence identity with the *Beauveria* strain inoculated (data not shown). No bands corresponding to

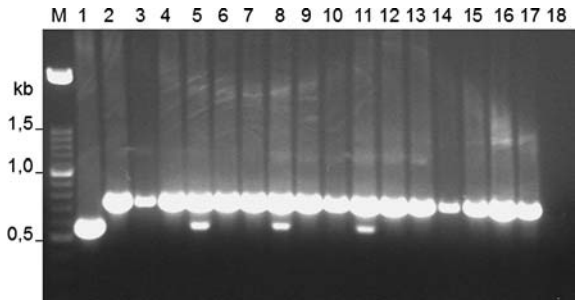

Figure 2. Agarose gel electrophoresis of the ITS1-5.8S-ITS2 PCR products from DNA extracted from opium poppy plants treated or untreated with a suspension of 7.5×10^8 spores/ml of *Beauveria bassiana* strain EABb 04/01-Tip and sampled at 24 (lanes 4–6), 48 (lanes 7–9), 72 (lanes 10–12) and 144 (lanes 13–18) hours after treatment. Lane 1, 0.1-kb DNA ladder XIV size marker (Roche Diagnostics, Mannheim, Germany); lane 2, Control DNA from conidia of *B. bassiana*; lanes 3 and 4, Control DNA from surface sterilised opium poppy seeds and seedlings grown under sterile conditions; lanes 4, 7, 10 and 13, control plants; lanes 5, 8, 12, 14 and 16, leaf pieces of opium poppy sprayed with a conidial suspension; lanes 6, 9, 11, 15 and 17, leaf pieces of opium poppy sprayed with a conidial suspension and surfaced sterilised; lane 18, blank.

B. bassiana were detected in control plants at any sampling time (Figure 2, lines 4, 7, 10 and 13). Using a similar molecular approach, *B. bassiana* was detected colonizing tomato seedlings [10] after seed application. However, in this study DNA was extracted from the whole tomato seedling, and no information related to tissue sterilisation prior to PCR analysis was provided. The lack of amplification of *B. bassiana* DNA in treatments where the fungus was inoculated is due probably to the sampling procedure (leave piece samples were taken randomly), and also the lack of specificity of the universal primer pair used [9] together to the greater abundance of plant DNA in the dilution used for PCR probably accounts for the preferential amplification of opium poppy DNA and the difficulty to amplify the fungal DNA.

Figure 3a shows the opium poppy leaf surface after being sprayed with *B. bassiana*. SEM images revealed that although numerous conidia were observed on the leaf surface, few germinated and penetrated it (Figure 3b). From our estimation of the number of conidia germinating and penetrating the leaf surface, it was obtained that 6–8% of conidia germinated, and fewer than 5% penetrated the leaf surface directly. Whilst there were few germ tubes elongating into hyphae and spreading across the leaf surface, very often a germ tube elongated only a short distance before ceasing its elongation and penetrating the leaf surface. We observed no hyphae growing into stomata; it seemed that the most common method of penetration into the leaf interior was through the epidermis (Figure 3b).

A cross-section of the cell wall around a penetration hole formed by a *B. bassiana* hypha showed that there was a narrow-neck region and a distortion of the plant cell cuticular ultrastructure. Penetration of the epidermal cell wall shows that the plant cell wall is completely breached, and the hyphae grow through the hole (Figure 4a and b). Whilst intracellular colonisation by *B. bassiana* was not observed, some hyphae were detected growing through the xylem vessels (Figure 4c–e). *B. bassiana* was also shown to grow through the corn xylem vessels, which could enable the fungus to travel within the plant, and ultimately to provide overall insecticidal protection [6]. In corn, extensive mycelial growth and branching of *B. bassiana* on the leaf surface was observed [6], while in opium poppy the characteristic form of germination was the production of only relatively short hyphal growth followed by cuticular penetration. The penetration of opium poppy by *B. bassiana* presented some similarities to events in corn [6] since we did not detect any appresorium-like structure, and the penetration occurred through a small hole. We observed no emergence of *B. bassiana* from or conidial formation on the surface of opium poppy leaves or stems. Whether *B. bassiana* completes a life cycle in the opium poppy plants or not remains unclear, since the crop has not been studied for fungal growth or conidial formation. In addition, it must be emphasised that the presence of the insect host within the plant may allow the fungus to complete its life cycle and may also contribute to horizontal transmission of the endophyte. We have demonstrated that a *B. bassiana* strain isolated from stem-borer larvae of *T. papaveris* attacking opium poppy can colonise the plant endophytically. A first series of experiments under field conditions show promise for this fungal strain to suppress these larvae systemically and to provide some

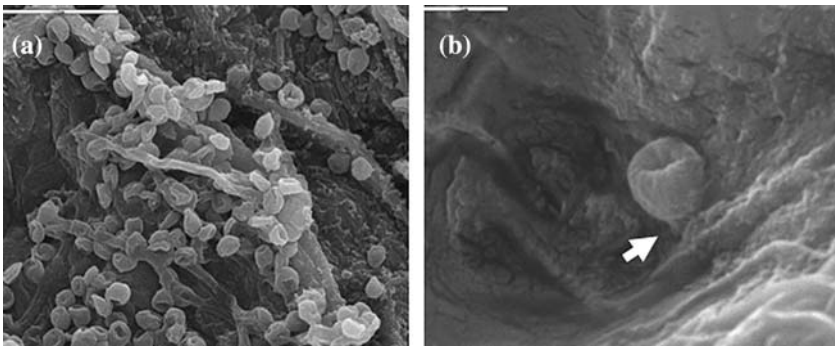

Figure 3. (a) Leaf surface and *Beauveria bassiana* conidia. Bar, 10 μm. (b) Germinating conidia with very short germ tube and penetration site. Bar, 2 μm.

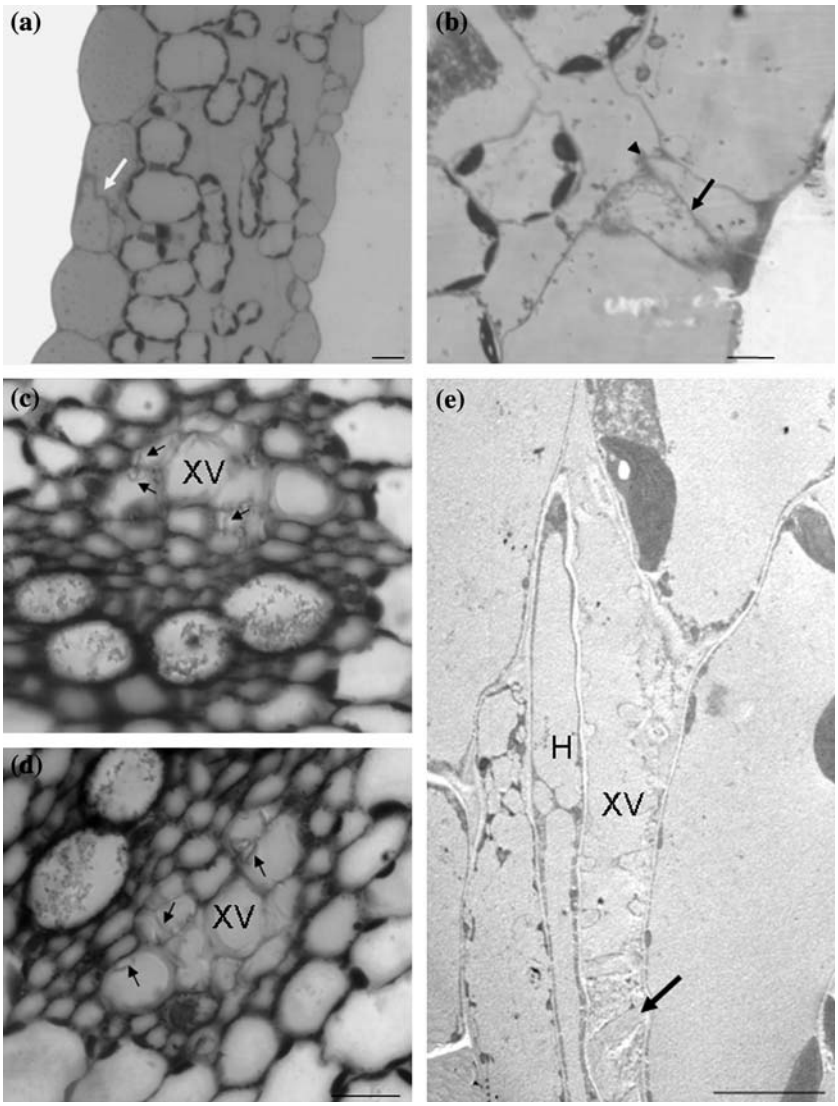

Figure 4. (a) Light micrograph of an opium poppy leaf epidermal cells showing penetration by *B. bassiana* (arrow). Bar, 100 μm. (b) Light micrograph of penetration of a leaf epidermal cell showing wall undulation (arrowhead). Bar, 100 μm. (c and d) Light micrographs of a leaf vascular bundle showing *B. bassiana* hyphae within the xylem vessel (arrows). Bars, 100 μm. (e) Electron micrograph of the leaf xylem vessel showing a glancing section through a hypha of *B. bassiana*. Bar, 5 μm. H, Hypha; XV, Xylem vessel. All micrographs have been taken 48 h after *B. bassiana* inoculation.

degree of season-long suppression of this plant's insect pests.

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