Journal of Invertebrate Pathology 114 (2013) 128–138

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

In-planta detection and monitorization of endophytic colonization by a *Beauveria bassiana* strain using a new-developed nested and quantitative PCR-based assay and confocal laser scanning microscopy

B.B. Landa [a,1], C. López-Díaz [b,1], D. Jiménez-Fernández [c], M. Montes-Borrego [a], F.J. Muñoz-Ledesma [d], A. Ortiz-Urquiza [b], E. Quesada-Moraga [b,*]

[a] Institute for Sustainable Agriculture (IAS), Spanish National Research Council (CSIC), Campus de Excelencia en Agroalimentación, CeiA3, P.O. Box 4084, 14080 Córdoba, Spain
[b] Department of Agricultural and Forest Science and Resources, Campus de Rabanales, University of Córdoba (UCO), CeiA3, P.O. Box 3048, 14080 Córdoba, Spain
[c] Department of Agronomy, Campus de Rabanales, University of Córdoba (UCO), CeiA3, P.O. Box 3048, 14080 Córdoba, Spain
[d] ALCALIBER I+D+i S.L.U., Ctra. Carmona-El Viso del Alcor, km 1.8, Carmona, Sevilla, Spain

ARTICLE INFO

Article history:
Received 18 February 2013
Accepted 24 June 2013
Available online 10 July 2013

Keywords:
Confocal laser scanning microscopy
Detection limit
Fungal endophytes
Papaver somniferum
Asymptomatic infection

ABSTRACT

Beauveria bassiana strain 04/01-Tip obtained from larvae of the opium poppy stem gall *Iraella luteipes* endophytically colonizes opium poppy plants and protect it against this pest. Development of a specific, rapid and sensitive technique that allows accurately determining the process and factors leading to the establishment of this strain in opium poppy plants would be essential to achieve its efficient control in a large field scale. For that purpose in the present study, species-specific primers that can be used in conventional or quantitative PCR protocols were developed for specifically identification and detection of *B. bassiana* in plant tissues. The combination of the designed BB.fw/BB.rv primer set with the universal ITS1-F/ITS4 primer set in a two-step nested-PCR approach, has allowed the amplification of up to 10 fg of *B. bassiana*. This represented an increase in sensitivity of 10000- and 1000-fold of detection than when using the BB.fw/BB.rv primers in a single or single-tube semi-nested PCR approaches, respectively. The BB.fw and BB.rv primer set were subsequently optimized to be used in real time quantitative PCR assays and allowed to accurately quantify *B. bassiana* DNA in different plant DNA backgrounds (leaves and seeds) without losing accuracy and efficiency. The qPCR protocol was used to monitor the endophytic colonization of opium poppy leaves by*B. bassiana* after inoculation with the strain EABb 04/01-Tip, detecting as low as 26 fg of target DNA in leaves and a decrease in fungal biomass over time. PCR quantification data were supported in parallel with CLMS by the monitoring of spatial and temporal patterns of leaf and stem colonization using a GFP-tagged transformant of the *B. bassiana* EABb 04/01-Tip strain, which enabled to demonstrate that *B. bassiana* effectively colonizes aerial tissues of opium poppy plants mainly through intercellular spaces and even leaf trichomes. A decline in endophytic colonization was also observed by the last sampling times, i.e. from 10 to 15 days after inoculation, although fungal structures still remained present in the leaf tissues. These newly developed molecular protocols should facilitate the detection, quantification and monitoring of endophytic *B. bassiana* strains in different tissues and host plants and would help to unravel the factors and process governing the specific endophytic association between opium poppy and strain EABb 04/01-Tip providing key insights to formulate a sustainable strategy for *I. luteipes* management in the host.

1. Introduction

Mitosporic ascomycetes traditionally known for their entomopathogenic characteristics, such as *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales), *Isaria* spp., and *Lecanicillium* spp. (Ascomycota: Hypocreales), have recently been reported as plant endophytes living internally within the tissues of some host plants without causing visible signs of infection (Quesada-Moraga et al., 2006a; Vega et al., 2008, 2009). Even if endophytes may be whether neutral, detrimental or beneficial to the host plant, the latter seems to be the feature for entomopathogenic ascomycetes protecting the plants against insects, nematodes, and even plant pathogens (Jaber and Vidal, 2010; Ownley et al., 2010). Recently, we have described such an endophytic association of the

* Corresponding author. Fax: +34 957218475.
E-mail address: equesada@uco.es (E. Quesada-Moraga).
[1] These authors contributed equally to this work.

0022-2011/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jip.2013.06.007

entomopathogenic fungus *B. bassiana* (Balsamo) Vuill. (Ascomycota: Hypocreales) and opium poppy *Papaver somniferum* L. (Quesada-Moraga et al., 2006a, 2009).

The opium poppy is one of mankind's oldest medicinal plants. This annual plant is today the commercial source of important narcotic analgesics such as morphine, thebaine, oripavine and codeine, which are extracted from its capsules. This crop has a global area of around 200,000 hectares (INCB, 2012) and its production is threatened by several pests, mainly due to the poppy stem gall wasp *Iraella luteipes* (Thompson) (=*Timaspis papaveris* Kieffer in Goury and Guignon 1905) (Hymenoptera: Cynipidae) (Chevin and Janvry, 1980; Sedivy and Chilar, 2005).

In a survey of natural enemies of *I. luteipes* in southern Spain conducted in 1995, a strain of *B. bassiana* was found infecting *I. luteipes* larvae within the stems of opium poppy plants. This strain named EABb 04/01-Tip was shown to be capable of becoming endophytically established in the plant when sprayed on leaves and to protect the crop at field conditions against the gall wasp (Quesada-Moraga et al. 2006a, 2009). Determining the process and factors that condition the establishment of this strain in opium poppy plants is essential to be able to use it in a large field scale for efficient control of the stem gall wasp. Although some preliminary attempt was done to detect the process of this endophytic association in the past (Quesada-Moraga et al., 2006a), many aspects of this association remain unknown.

Unraveling mechanisms mediating plant host-fungal entomopathogen recognition, establishment and colonization dynamics is possible if the presence of the fungus in the crop plant and/or in other plant species that can serve as sources of inoculum and in other pathogen habitats, is detected, differentiated and even quantified. Furthermore, as recently proposed for plant pathogens (Narayanasamy, 2011), detection of entomopathogenic fungal endophytes in crop plants and other host plant species and also in the environment such as soil, water and air, is essential and may be required in order to: (i) determine the presence and quantity of the endophyte in a crop to ascertain its preventive or curative potential; (ii) elucidate the mechanism of plant colonization by the endophyte and ascertain whether it is vertically transmitted; (iii) assess the impact of plant production and protection practices on the endophyte natural inoculum levels; (iv) verify fungal inoculation of seeds and planting materials; (v) monitor the endophyte population in the agroecosystem; and (vi) assess the possible *in planta* interactions among natural and released strains of fungal endophytes.

Methods frequently used to estimate fungal occurrence within plant tissues, such as direct visualization by different microscopy techniques and plating on culture media not only are laborious and time consuming but also they may overlook fungal occurrence due to uneven distribution of fungal nuclei (from zero to several thousand per cell) or due to physiological activity or vitality of hyphae. Likewise, fungal species that do not grow or grow very slowly in culture media may be-overlook, while species that grow well in culture may be over-represented. To solve some of those limitations development of PCR-based molecular methods for specifically detecting and monitoring the endophyte of study can be used; however, these may also present other weaknesses (i.e. they do not allow monitoring the exact location of the endophyte in plant tissues). Combination of quantitative and qualitative data achieved by both molecular and microscopy methods are probably the best choice to monitor endophytes, with the advantages of each technique complementing the drawbacks of the other.

Here we describe a combination of molecular and microscopy techniques to ascertain the mechanisms of opium poppy leaf colonization by EABb 04/01-Tip strain. First, we developed and optimized a nested-PCR protocol to detect and monitor the fungal strain within the plant tissues. We then implemented the designed primers to be used in a real-time quantitative PCR (qPCR) protocol to assess the biomass of the endophytic strain that established within plant tissues. Finally, we monitored the exact biomass and location of the strain in a time course experiment when endophytically established in plant tissues by combining the developed qPCR protocol and confocal laser scanning microscopy (CLSM) using a labeled green fluorescent protein (GFP) 04/01-Tip strain.

2. Materials and methods

2.1. Fungal isolates

The *B. bassiana* strain EABb 04/01-Tip isolated from dead *I. luteipes* larvae at a field in Carmona (Seville) and shown to behave as an endophytic strain (Quesada-Moraga et al., 2006a) when inoculated into opium plants, was used throughout all the study (Table 1). This strain is deposited at the C.R.A.F. University of Córdoba Entomopathogenic Fungi Collection, Córdoba, Spain, and at the Spanish Collection of Culture Types (CECT), University of Valencia, with accession No. CECT20744. For the CLSM study the strain was transformed with the GFP following the efficient *Agrobacterium*-mediated transformation for *B. bassiana* protocol described in Fang et al. (2004). To check for the successful expression of GFP, single-spored cultures of transformants were examined with a confocal laser microscope (Nikon TE 2000-S, Melville, NY, USA). One out of nine transformants showing appropriate fluorescence emission and stability was selected to carry out the bioassay. Monosporic cultures of the wild and GFP-transformed strain were grown on slants of Malt Agar (MA; Biocult, Madrid, Spain) at 25 °C in the dark and stored at 4 °C.

Additionally for designing the specific *B. bassiana* protocol a collection of 47 fungal isolates were used (Table 1). Those isolates represent 37 strains of *B. bassiana* isolated from different insects (16 strains), soil (five strains), phyllosphere of different plants (7 strains), or behaving as endophytic isolates in grasses (nine strains; Herrero et al., 2012); nine isolates of other fungal species including *Metarhizium guizhouense* (one isolate), *Rhizoctonia solani* (one isolate), *Verticillium albo-atrum* (one isolate), *Verticillium dahliae* (one isolate), *Fusarium globosum* (one isolate), *Fusarium anthophilum* (one isolate), *Fusarium avenaceum* (one isolate), *Fusarium andiyazi* (one isolate), and *Fusarium circinatum* (one isolate). Additionally, a DNA sample extracted from opium poppy seeds infected by the oomycete and obligate biotroph *Peronospora arborescens* (one specimen), the causal agent of the downy mildew in opium poppy (Montes-Borrego et al., 2009) was used.

Single-spore cultures of all isolates are deposited in the culture collections of the C.R.A.F. University of Córdoba Entomopathogenic Fungi Collection, Córdoba, Spain or the Department of Crop Protection, Institute for Sustainable Agriculture, Spanish National Research Council, Córdoba, Spain. Active cultures of the *B. bassiana* isolates were obtained on MA, whereas active cultures of the remaining fungal isolates were obtained on potato dextrose agar (PDA; Difco Laboratories, Detroit, Michigan, USA).

For fungal DNA extraction, actively growing cultures were placed onto a film of sterile cellophane layered over a plate of Sabouraud Dextrose Chloranphenicol Agar (SDCA; Biocult, Madrid, Spain) for *B. bassiana* isolates or on PDA for the remaining isolates. Inoculated plates were incubated for 5–7 days at 25 °C in the dark. Then, mycelia growing over the cellophane surface were scraped directly with a sterile scalpel, lyophilized, and stored at −20 °C until used. Total DNA from opium poppy seeds infected by *P. arborescens* was the same than that used in Montes-Borrego et al. (2009).