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Method Article

Method for observing *Beauveria bassiana* colonization in plants

Qiu-yang Wei[a,*], Yue-ni Liu[b]

[a] *Chongqing Academy of Chinese Materia Media, China*
[b] *College of Plant Protection, Northwest A & F University, China*

A B S T R A C T

Microbes interact in a multitude of ways with host plants, can dwell as endophytes within plants causing no apparent disease, and often provide benefits to their host. Observing microorganism distribution and colonization is a prerequisite for interactive research. To this end, we describe use of fluorescent staining for microorganism labeling and highlight its simplicity, and efficiency. Fluorescein can quickly bind to *Beauveria bassiana* spores, producing bright green fluorescence that can be observed even inside plant tissues. This method provides an intuitive visual image that can be utilised for subsequent data acquisition and statistical analysis.

- Our protocol depends on binding of fluorescein diacetate (FDA) specifically to microorganisms. The fungus hydrolyses and metabolises FDA in cells to produce bright green fluorescent products. This fluorescent signal can easily penetrate plant epidermis and be detected by fluorescence microscopy.
- FDA, which itself does not emit light, will emit a fluorescent signal only when combined with *B. bassiana.* Concomitant genetic testing of the fungal ITS confirmed the high level of the fluorescent staining method for detection of *B. bassiana.*
- Compared with the previous green fluorescent protein (GFP) labeling methods, this protocol improved the labeling efficiency of microorganisms and simplifies the process.

A R T I C L E I N F O
Method name: Fluorescent labeling procedure for *Beauveria bassiana*
Keywords: Fluorescence, Fluorescein diacetate (fda), *Solanum lycopersicum*, *Beauveria bassiana*, Endophytes
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Abbreviations: fda, fluorescein diacetate; GFP, green fluorescent protein.

* Corresponding author.
E-mail address: qiuyangwei2018@163.com (Q.-y. Wei).

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Specifications Table

Subject Area	Agricultural and Biological Sciences
More specific subject area	Entomogenous fungi interacting with host plants
Method name	Fluorescent labeling procedure for *Beauveria bassiana*
Name and reference of original method	We labelled *Beauveria bassiana* spores with fluorescein and observed their distribution throughout plant tissues. This protocol is simple, intuitive, and efficient for observation of *B. bassiana* spores. Originally, the protocol was developed by Jones and Deng-jie. Jones and Sneft (1985) [2]. An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. Journal of Histochemistry Cytochemistry. 33(1): 77–79. DOI: 10.1177/33.1.2578146 Deng-jie et al. (2015) [3]. A new fluorescent microscopy method for identifying *Beauveria bassiana* infected *Bemisia tabaci* nymphs. Chinese Journal of Applied Entomology. 52(1): 267–271. DOI: 10.7679/j.issn.2095-1353.2015.028
Resource availability	

Method details

Background

Observing the colonization and distribution of microorganisms within plants is important in studies on plant–microbe interactions [1]. Individual microbes are generally small and impossible to observe with the naked eye. Those within a plant organism are especially hard to detect. Fluorescein diacetate (FDA) is a non-polar ester, which can be hydrolysed by esterases in living cells, with no toxic effects on the organism. The resulting hydrolysed product can be excited as bright green fluorescence by 450–490 nm light [2,3]. This facilitates visualization of the spores inside the plant tissue. Compared with the traditional green fluorescence protein (GFP) labeling, the FDA labeling method is greatly simplified [4].

Required reagents and equipment

Fluorescein diacetate (FDA)
Potato dextrose agar (PDA)
Acetone
Deionised water
Tween-80
Sodium hypochlorite
Beaker
Graduated cylinder
Petri dish
Artificial climate chamber (RDN-260A; Ningbo Dongnan)
Haemocytometer
Fluorescence microscope (OLYMPUS BX51)

Preparation

Fungi and plants

A *Beauveria bassiana* strain (Bb252), maintained at Biotechnology center, Southwest University, was stored at −80 °C. The strain was originally isolated in 2015 from *Chilo suppressalis* on maize in Yongchuan District, Chongqing, China, and was separated into single spores that were used for molecular identification. Before the tests were initiated, Bb252 was grown at 28 ± 1 °C on potato dextrose agar medium for 9 days in the dark. The conidia were harvested by gently scraping the surfaces of agar slants flooded with 10 mL 0.1% Tween-80 sterile water. A haemocytometer was used to determine the final concentration of the spores.

The seeds of tomato (*Solanum lycopersicum*) Hezuo 903 were purchased from Changzhong Tomato Co., Ltd, (Shanghai, China). Standard sterilization procedures were followed during the tests. Seeds

were surface sterilised with 1% NaClO for 5 min, rinsed three times with sterile distilled water, and dried on sterile filter paper [5]. After that, the seeds were directly sown in a sterile nutrient soil (soil was sterilised at 75 °C for 24 h; organic matter 463 g/1000 g, $N + K + P = 5.36$ mg/1000 g, trace element 1.7 μg/1000 g; Hengoda Fertilizer Technology Co., Ltd, Lianyungang, China) and germinated in an artificial climate chamber (14 h light and 10 h dark photoperiod, 26 ± 1 °C, 75 ± 5% relative humidity). Plants were used in the experiments when they reached 20 cm in height.

Experimental procedure

1. A fluorescent dye stock solution was prepared by dissolving 4 mg FDA in 1 mL acetone and stored at 4 °C protected from light.
2. Before the experiment, 35 μL of FDA stock solution was diluted in 4 mL of deionised water to prepare a fluorescent dye working solution.
3. Plants were sterilised according to the above seed disinfection method.
4. The spore suspension (1×10^7 conidia/mL) and fluorescent dye working solution were mixed in a 1:1 ratio.
5. Plant tissue was soaked in the spore suspension for 10 s and then stored at 4 °C protected from light for 0 h and 72 h.
6. The plant tissue was again disinfected 72 h after the fungal treatment to eliminate spores on its surface.
7. The samples were observed under a fluorescence microscope (450–490 nm).

Note: All procedures were carried out at low temperature and in the dark.

Method validation

The plant tissues soaked in *B. bassiana* spore suspension were observed after surface disinfection. Plant tissues not inoculated with *B. bassiana* did not produce fluorescent signals when mixed with FDA (Fig. 1). The subsequent results confirmed the induction of bright green fluorescent signal inside plant tissues (Fig. 2). The distribution of the green signal was not limited to a certain structure. Interestingly, the fluorescence signal was observed in the mesophyll, the stem, and even in the leaf veins of tomato plants. It is reasonable to infer that these spores can be transported via the vessels inside plant tissues. These images allowed us to discern the distribution and diffusion of *B. bassiana* throughout the plants. This method facilitates the follow-up-identification of microbes and the research on microbe–host interactions.

To verify the accuracy of the fluorescent labeling of *B. bassiana*, we collected the previously FDA-labelled samples with confirmed fluorescent signal for fungal genetic verification. Fresh plant tissue (0.5 g) was ground in liquid nitrogen, and *B. bassiana* genomic DNA was extracted with a BioSpin Fungal Genome DNA Assay Kit (BIOER, Hangzhou, China). DNA fragments were amplified by a two-step nested PCR protocol using ITS1-F and ITS-4 primer pair in the first PCR and Bb.fw and Bb.rv primer pair in the second PCR (Table 1).

Each reaction volume (25 μL) included 0.5 μL each of 10 mM forward and reverse primer, 22 μL T3 Super PCR Mix (TsingKe, Shanghai, China), and 2 μL DNA template. The reaction protocol was as follows: an initial step at 98 °C for 2 min, 35 cycles of 98 °C for 10 s, 61 °C for 10 s, 72 °C for 10 s, and a final extension at 72 °C for 2 min; the second PCR using nested primers was conducted utilising the

Table 1
Sequence information of primers used in two-step nested PCR.

PCR step	Primer name	Sequences
First step	ITS1-F	5′-CTTGTTCGCTATCGGTCTC-3′
	ITS-4	5′-TCCGTAGGTGAACCTGCGG-3′
Second step	Bb.fw	5′-GAACCTACCTATCGTTGCTTC-3′
	Bb.rv	5′-ATTCGAGGTCAACGTTCAG-3′

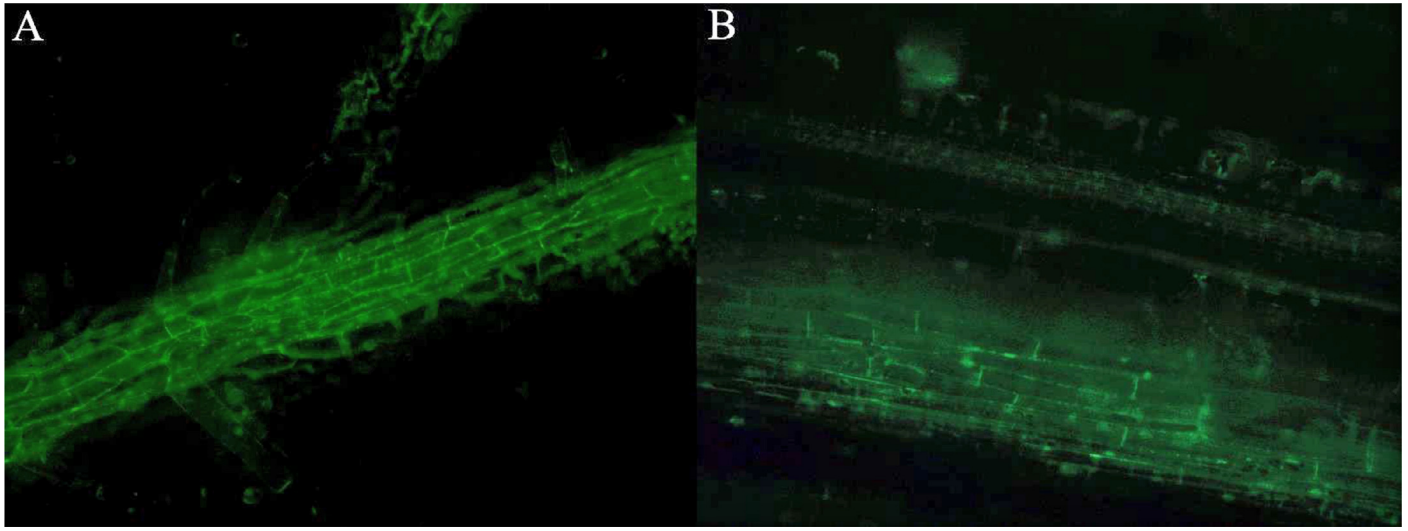

Fig. 1. Observation of plant tissues not inoculated with *Beauveria bassiana* inoculated.
A: Plant leaves; B: Plant stem. No fluorescence signal confirms the absence of self-luminescence of plants.

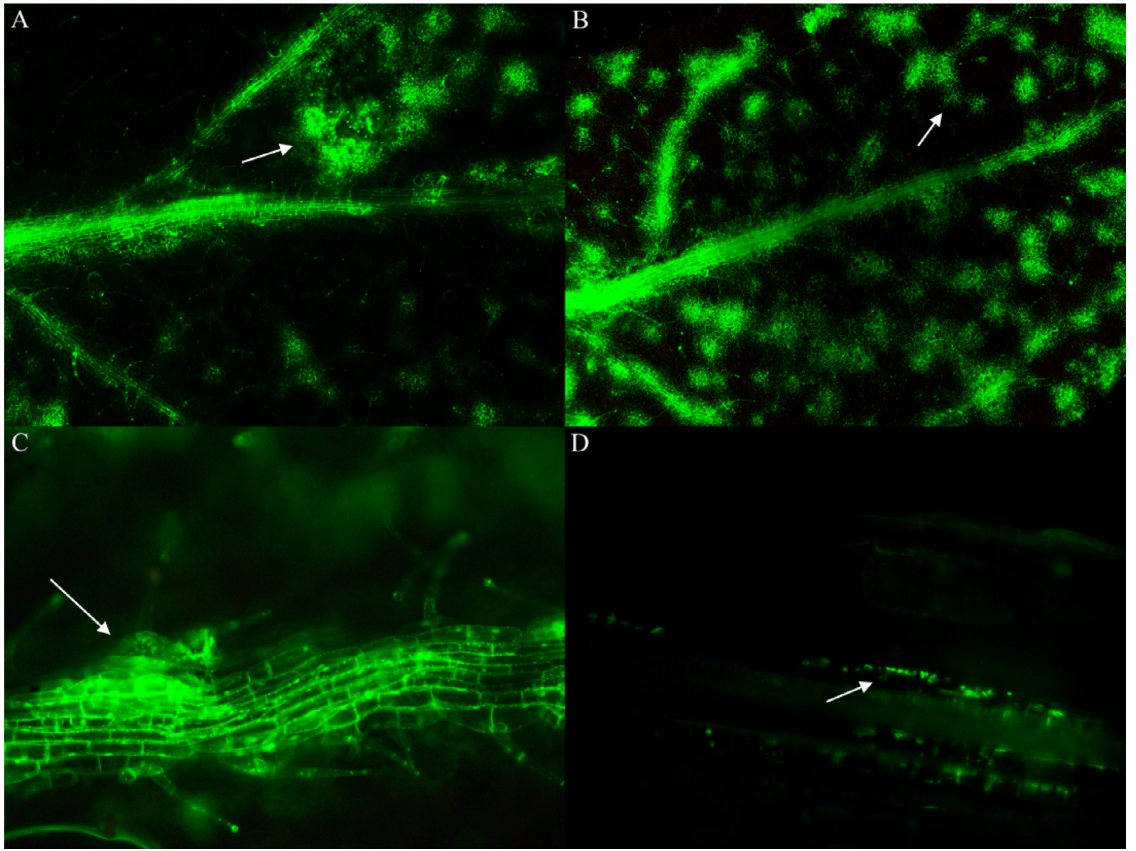

Fig. 2. Observation of *Beauveria bassiana* in plant tissues.
A and B, tomato mesophyll tissue; C and D, tomato stalk tissues. The bright green fluorescence indicates *B. bassiana* marked by fluorescein diacetate (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

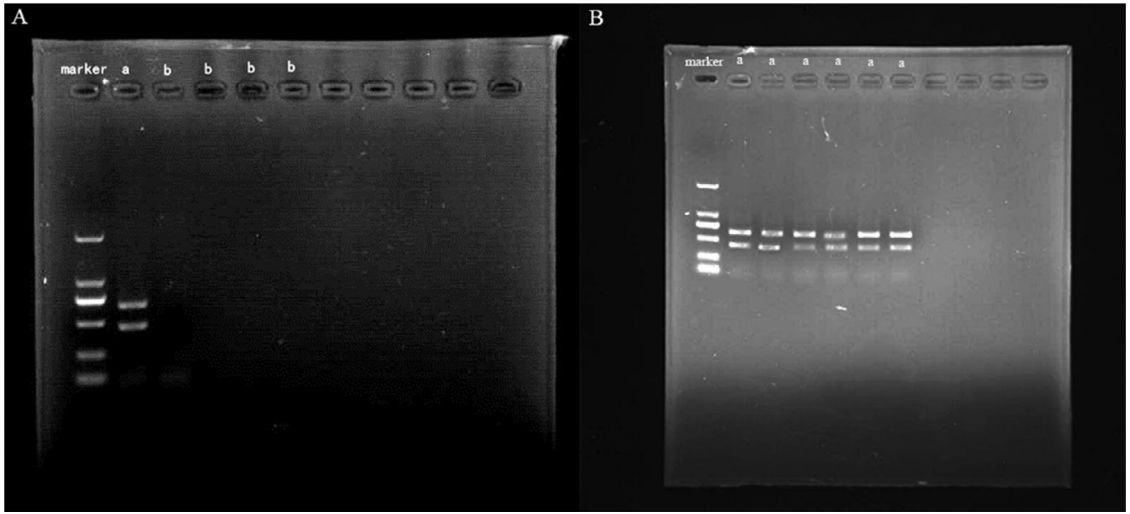

Fig. 3. Detection of *Beauveria bassiana* sequences by PCR in plant tissues.

same parameters, except for the annealing temperature, which was set to 55 °C for 10 s [5,6]. The PCR product was detected by electrophoresis and its base sequence was determined by Tsingke Biological Technology Co., Ltd. Finally, the amplified *B. bassiana* sequence samples were matched to the samples that emitted fluorescence signal.

Before analysing the fluorescence results, we verified that the disinfection step, implemented immediately after the treatment of sterilised plants with spore suspension, provided a positive sterilization effect. This was done by PCR amplification of the extracted fungal genome. Validation studies using nested PCR did not detect any *B. bassiana* sequences in four non-inoculated control samples (Fig. 3A). This confirmed that the plant surface disinfection procedure used in this study effectively eliminated the interference with *B. bassiana in vitro*. The sequences of *B. bassiana* were amplified in six plant tissues that were also shown to emit fluorescence signals (Fig. 3B). These results demonstrate the high detection accuracy of the fluorescence method.

A: detection of *B. bassiana* in surface-sterilised tomato plants; B: detection of *B. bassiana* colonization in inoculated tomato plants.

Lower case letter a: plant tissue soaked with *B. bassiana* spore suspension; Lower case letter b: samples that were immediately sterilized after immersion in *B. bassiana* suspension and released no excitation light after fluorescence detection.

Our experimental results confirmed the high accuracy of fluorescent labeling for microbe detection. This method can be used to clearly observe fungal distribution in multiple tissues of a plant. Although this technique is suitable for qualitative research on the distribution of fungi, we anticipate that these findings will be of interest to investigators in their studies on the interactions between microorganisms and plants.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2021.101364.

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