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Short communication

Use of a green fluorescent protein marker for studying splash dispersal of sporangia of *Phytophthora infestans**

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Abstract

The purpose of this study was to develop a new technique to evaluate the number of spores incorporated in splash droplets by the use of an engineered fluorescent pathogen strain and image analysis hardware and software. The inoculum source consisted of a tomato leaflet infected with a *Phytophthora infestans* strain transformed with the gene encoding the green fluorescent protein (GFP). Splash droplets formed after impact of incident drops on sporulating lesions were collected on microscope slides located at different distances from the source. Each slide was examined using a fluorescent microscope to visualize the GFP expressing sporangia. Photographs were taken and assessed using image processing to count the sporangia incorporated in each droplet. Data analysis confirmed characteristics of splash dispersal shown using other methods. The use of fluorescent sporangia also facilitates the selective detection and counting of viable (living) sporangia, and is a tool that can be use in the study of splash dispersed diseases.

Splash droplets generated by raindrop impaction on plants are known to be the dispersal agent of numerous micro-organisms (fungi, oomycetes and bacteria) within crop ecosystems (Madden, 1992). Several techniques of counting spores in splash droplets have been investigated. Most of them are derived from Gregory et al. (1959) and are based on collecting droplets carrying spores on glass slides and counting by sight under a microscope. These techniques are tedious, time consuming and require specific spore recognition skills because of airborne particles such as dust, other fungal spore structures, or pollen that can contaminate the slides. The addition of dye on the slide or on the leaf to visualize particles has also been used (Yang et al., 1992), but additives can alter the interaction of the spores with plant surfaces and/or the impacting drops, thus altering the splash-dispersal process. Fitt et al. (1982) proposed an improved technique using photographic film coupled with the use of semi-automatic image analysis. This image analysis led to an approximate reduction of 30% in counting time and analysis, but a person was still needed to count spores and to identify spores on the digital picture. Elaborate techniques of image analysis have also been successful for quantifying emission of pollen or other small particles (Morris, 1995); these techniques are limited by the difficulty of introducing biological parameters into the image analysis software, such as species differences or information on the viability of the counted particles.

In this study we describe a new approach developed for analysis of splash dispersed pathogens. An engineered fluorescent pathogen strain

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and a fully automated image analysis system were used to observe and quantify the pathogens incorporated in splash droplets. The engineered plant pathogen was transformed with the *gfp* reporter gene that expresses the green fluorescent protein (GFP), a naturally occurring fluorescent protein from the jellyfish *Aquorea victoria* (Si-Ammour et al., 2003). GFP is particularly appropriate for assaying living cells since fluorescence does not require the addition of chemical substrates (Bottin et al., 1999). Plant pathogenic oomycetes and fungi expressing GFP have been used to measure pathogen biomass, visualize infection structures, and estimate gene expression in infected plant tissue (van West et al., 1999).

Here, we focused on the potato and tomato late blight pathogen *Phytophthora infestans*. Infected tissue necrotizes and the mycelia develops sporangiophores that emerge through the leaf stomata to produce numerous sporangia. It is notable that sporangia of species of *Phytophthora* are dispersed only by rain (Madden, 1992; Yang et al., 1992).

We used a pathogenic transformant of *P. infestans* strain 208M2, that expresses high levels of GFP (Si-Ammour et al., 2003). Zoospores of *Phytophthora infestans* were collected from colonies grown on rye-sucrose agar Petri dishes, at 18–21 °C for 10–15 days. To induce zoospore production the plates were filled with cold (4 °C) sterile distilled water and kept for 2–3 h at 4 °C. The delivery of zoospores starts in 20 min and continues for 2–4 h. The inoculum suspension (about 20,000 zoospores ml^{-1}) was sprayed on mature tomato leaves. Mature tomato leaves (cultivar oh7814) were collected from plants grown in the greenhouse for 5 weeks in August–September under natural light conditions of 10 h daylight. After inoculation, the leaves were kept in moisturized sealed boxes with a transparent lid in a clean room at 18 °C and 12 h of artificial light (2–60 W fluorescent bulbs). Five days post inoculation the infected area was covered with sporangia. The inoculation set-up created a uniform sporulation pattern and all leaf surfaces were assumed to have produced the same quantity of sporangia at a given time after the inoculation.

The sporulating leaves were placed on a support at ground level. Incoming water drops were generated from a vertically-held hypodermic needle to simulate rain. Drops (diam 2.70 and 3.30 mm) released from two different heights (1.0 and 1.5 m) fell onto the infected leaf. Drop diameters were assessed by weighting. Splash droplets were collected on five pairs of microscope slides (25.4×76.7 mm) located at the same height as the leaf. Each pair of slides was put side by side along the shortest length and the centre of each pair of slides was located respectively at 10, 45, 81, 116 and 151 mm from the source. The experiment was repeated 3 times for each drop fall height tested. As a genetically modified organism, the experiment was carried out in a confined room that provides UV-light for sterilization.

The Leica DMIRB fluorescent microscope with the digital camera and image acquisition software Magnifyr by Optronics were used for image acquisition from the microscope slides. The microscope offers the ability to visualize and electronically capture images over time. The images were taken at a 1280×1024 pixel resolution, with a colour depth of 2^{24}. This system provides a very bright light source for excitation of fluorescent dyes or proteins (GFP, etc.). The light excitation source for GFP green light enhancement was achieved using a filter (I32) for excitation at 450–490 nm with light emission at 515 nm.

After picture acquisition, the images were analyzed using software 'ImageJ' (freely available at http://rsb.info.nih.gov/ij/). The resulting image had a black background with green spots corresponding to viable (i.e. living) sporangia (Figure 1). It was determined that the software would read any green shaped spot, a single sporangium or a clump of sporangia. The software automatically recorded the area of each spot. In the case of a clumping of sporangia (where individual spores could not be discerned), the total green area was compared with the mean area of a single sporangium, and an estimate of the number of sporangia in the clump was calculated. Fortunately clumps of sporangia were rare in our experiments.

In preliminary studies, we compared images with fluorescent light and without, in order to check the reliability of the technique. Without fluorescent light, sporangia were counted by sight and compared with the count of sporangia under light excitation that enhanced the green fluorescence. Using the two counting methods for the same slides, the same numbers of viable sporangia were found; however damaged, i.e., non-viable sporangia did not fluoresce. Thus, the only difference found between both counting approaches was

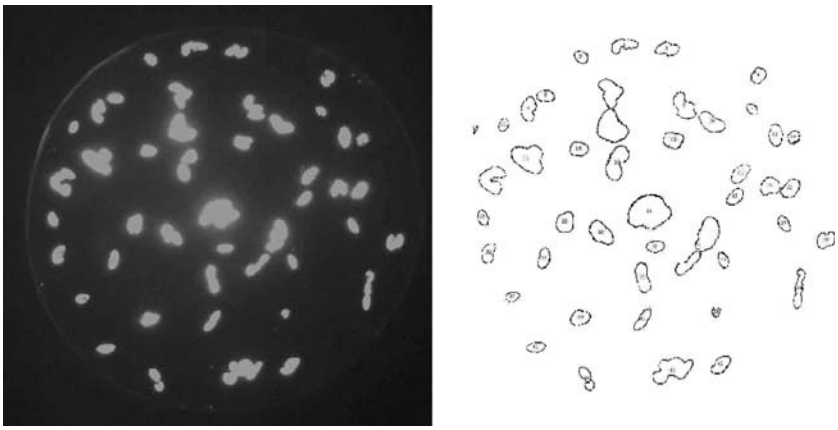

Figure 1. Schematic depiction of the stages in image analysis of water droplets on slides. Left: original fluorescent microscope image of water trace on glass slide containing sporangia of *Phytophthora infestans* (strain 208M2), which was transformed to express the GFP protein. A single sporangium has a 'lemon' shape 22 – 32 × 16 – 24 μm in dimension. Right: final image, after shape recognition, which allows for counting and labelling of sporangia (indicated by traces).

that non-viable sporangia could be observed by the direct visual counting method.

Compared to previous studies on the dispersal of spores due to the impact of water drops (Gregory et al., 1959; Fitt et al., 1982), the experimental setup described here provides fast, systematic and reliable assessment of the number of pathogen propagules (e.g. spores, sporangia) incorporated into splash droplets. The time for image acquisition and processing is considerably less than the visual screening. The entire image analysis and particle counting for one slide only takes the required time to put the slide under microscope (i.e. time of image processing is not significant), regardless of whether there were zero or 300 sporangia present. On the other hand, visual counting with the aid of a microscope depends on the number of splash droplets on the slide and the density of spores in the traces of the droplets (Yang et al., 1992). It could easily take several hours to count the spores on a single slide when there are many droplets and spores. Moreover, visual counting also requires skills in spore recognition, which is not trivial.

Unlike the situation when dyes are added to pathogen spores in dispersal studies, there is no concern about the presence of the genetic marker affecting properties of the leaves (such as surface tension), the surface of the pathogen spores, or the water on the leaf surface. The use of fluorescent sporangia facilitates the detection and counting of exclusively viable (epidemiologically significant) sporangia by image processing. Previously to distinguish living from dead pathogen propagules, selective media were used to test for germination (e.g. Madden, 1992) that can involve extra cost and work.

The same methodology can also be used to discriminate between species or isolates of pathogens. For instance, it is easier to track a specific pathogen strain in a mixture of strains (Pepperkok et al., 1999). Moreover, different markers which fluoresce at specific wavelengths such as red or blue (Jakobs et al., 2000) can be combined to study a complex of inocula composed by diverse organisms. The main limitation of the approach is the availability of a transformed strain of the microorganisim expressing GFP or other fluorescent proteins. These strains are now being produced more frequently, primarily for molecular investigations such as studying and quantifying aspects of disease development (Si-Ammour et al., 2003). Our study points to a new application for the use of the GFP marker in plant disease epidemiology in laboratory conditions.

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