Rapid Staining Method to Detect and Identify Downy Mildew (Peronospora belbahrii) in Basil

Authors: Adolfina R. Koroch, Thomas S. Villani, Robert M. Pyne, and James E. Simon
Source: Applications in Plant Sciences, 1(7)
Published By: Botanical Society of America
URL: https://doi.org/10.3732/apps.1300032
Rapid Staining Method to Detect and Identify Downy Mildew (Peronospora belbahrii) in Basil

Adolfina R. Koroch2,5, Thomas S. Villani3,4, Robert M. Pyne3, and James E. Simon3

2Science Department, Borough of Manhattan Community College, The City University of New York, 199 Chambers Street, New York, New York 10007 USA; 3New Use Agriculture and Natural Plant Products Program, School of Environmental and Biological Sciences, and the New Jersey Agricultural Experiment Station (NJAES), Rutgers, The State University of New Jersey, 59 Dudley Road, New Brunswick, New Jersey 08901 USA; and 4Department of Medicinal Chemistry, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, New Jersey 08854 USA

Premise of the study: Demand for fresh-market sweet basil continues to increase, but in 2009 a new pathogen emerged, threatening commercial field/greenhouse production and leading to high crop losses. This study describes a simple and effective staining method for rapid microscopic detection of basil downy mildew (Peronospora belbahrii) from leaves of basil (Ocimum basilicum).

Methods and Results: Fresh leaf sections infected with P. belbahrii were placed on a microscope slide, cleared with Visikol™, and stained with iodine solution followed by one drop of 70% sulfuric acid. Cell walls of the pathogen were stained with a distinct coloration, providing a high-contrast image between the pathogen and plant.

Conclusions: This new staining method can be used successfully to identify downy mildew in basil, which then can significantly reduce its spread if identified early, coupled with mitigation strategies. This technique can facilitate the control of the disease, without expensive and specialized equipment.

Key words: basil; downy mildew; light microscopy; Peronospora belbahrii; staining; visualization.
Although DNA-based assays such as PCR are suitable for the detection of obligate parasites in the laboratory setting (Belbahri et al., 2005; Farahani-Kofoet et al., 2012), these techniques are impractical for field identification purposes and require specialized expertise not available everywhere. Observation of pathogen structures under the microscope remains an effective tool for plant pathogen identification. A simple microscopic method for easy detection of downy mildew can be critical for proper disease management and epidemic prevention. Fluorescence differential staining methods that help the visualization of the pathogen and host plant tissues have been reported (Williamson et al., 1995; Hoch et al., 2005; Diez-Navajas et al., 2007). However, these methods require the use of fluorescence microscopes, as well as a certain level of expertise and multi-step procedures.

A simple, reliable staining method that provides high contrast for rapid and early detection of downy mildew can significantly reduce its spread, thus facilitating control of the disease. This technique was originally developed to allow plant breeders in our breeding program to quickly and inexpensively identify the presence of the pathogen not only in the greenhouse but also in the field trials throughout the course of basil downy mildew resistance trials. This procedure will benefit growers, breeders, disease managers, pathologists, and plant diagnostic clinicians because the pathogen can be detected under low magnification (as low as 10×), which will allow a prompt disease response. Early detection and response will allow for reduced spread of the pathogen. This study describes a simple and effective differential staining method for rapid microscopic detection and observation of basil downy mildew (\textit{P. belbahrii}) using leaves of sweet basil with minimal sample preparation.

**METHODS AND RESULTS**

**Plant material and pathogen inoculum**—Diseased plant material was obtained from a 2011 field trial at the Rutgers Agriculture and Research Extension Center (RAREC) in Bridgeton, New Jersey, and maintained in the Rutgers research greenhouse. Diseased leaves were washed with distilled water, and two drops of a suspension containing approximately \(5 \times 10^{4}\) spores/mL were used as inoculum. To maintain stock inoculum, host sweet basil (\textit{O. basilicum} cv. DiGenova Stokes Seed) seedlings were grown out in 7-d successions and repeatedly inoculated with spores harvested from infected plants.

**Fig. 1.** Basil leaf infected with \textit{Peronospora belbahrii}. (A) Distinct staining of characteristic branched sporangiophores and sporangia after one week of inoculation. (B) Direct germination of sporangia and penetration through stomata (arrows) 48 h after inoculation. (C) High resolution of differential staining of sporangiophores bearing elliptical sporangia. (D) Sporangiophores emerging from stomata.
Applications in Plant Sciences 2013 1(7): 1300032
doi:10.3732/apps.1300032

Koroch et al.—Staining method to identify downy mildew in basil

inoculation, plants were subjected to a 48-h period of 100% leaf wetness and relative humidity with temperatures between 22–24°C and 12-h/12-h light/dark cycle. Dense sporulation was observed 7 d after inoculation. The identity of downy mildew pathogen (P. belbahrii) was confirmed using a real-time PCR assay as previously reported (Belbahri et al., 2005).

Clearing and staining method—Fresh leaf sections (approximately 1 cm²) infected with P. belbahrii from the field trials and from plants artificially inoculated from the greenhouse were collected with a scalpel and placed on a microscope slide with the abaxial side facing up. Two drops of Visikol™ (Phytosys LLC, New Brunswick, New Jersey, USA) clearing solution were applied so as to completely submerge the sample, and a cover slip (0.17 mm thickness) was then applied. To clear the plant material, the microscope slide was placed on a hot plate for approximately 30–60 s until just before boiling, when air bubbles move out to the edges of the slide (Villani et al., 2013). After cooling, the cover slip was removed and two drops of stain were applied. The following four treatments were examined:

1. methylene blue (0.1% in water)
2. aniline blue (0.1% in water)
3. iodine solution (0.5 g I₂ plus 1.5 g KI)
4. iodine solution for 1 min followed by one drop of acid (glacial acetic acid, concentrated phosphoric, and 70% sulfuric acid were tested)

A cover slip was added on the stained samples. Each sample was replicated at least three times. All the microscopic image analyses were taken on a Nikon Eclipse 80i microscope, with NIS Elements D 3.00 SP7 Laboratory Imaging software (Nikon Instruments, Melville, New York, USA). After clearing the leaf with Visikol, the translucent pathogen asexual hyphae and light, elliptical sporangia were clearly observed. Methylene blue or aniline blue stains were nonselective between pathogen and plant tissues. The addition of iodine solution alone was not effective in staining the cell walls of P. belbahrii, but it did stain the starch granules in the guard cells from the basil epidermis. Only after adding two drops of sulfuric acid to the sample cleared with Visikol and stained with iodine solution did the pathogen become a dark brown color in contrast to the leaf tissue. This distinct staining of the pathogen on the surface of the leaf generated an image with high contrast between the pathogen and basil epidermis within 5 min (Fig. 1A). Iodine/potassium iodide stain is commonly used in microscopy to detect the presence of carbohydrates in different organisms or organs (Jackson and Snowdon, 1990). It has been reported that the chemical composition of the cell wall of Peronospora spp. is one of the characteristics that differentiate this organism from true fungi. The major component of Peronospora spp. cell walls is cellulose, β-(1, 3) glucans, and β-(1, 6) glucans (Bartnicki-Garcia, 1968). Other acids (acetic glacial and phosphoric acid) evaluated did not result in high-contrast coloration of the pathogen relative to the host (data not shown).

This method was originally developed as part of an ongoing effort to develop downy mildew–resistant basil plants. Using this simple staining method (protocol is summarized in Appendix S1), downy mildew was clearly and easily detected. Direct germination of sporangia was observed 48 h after inoculation. A single germ tube was observed per sporangium and could be followed to a stoma by microscope. Compared to molecular techniques such as PCR, this protocol describes an easier, less expensive, and more rapid technique that does not require sophisticated equipment to identify presence of the pathogen. This diagnostic capability allows for appropriate disease control measures to be taken as a function of the pathogen life cycle, which itself can be studied using the technique described in this paper. Early detection of the disease allows for control of its spread by allowing for the identification and quarantine or disposal of infected plants or the application of chemical control agents to reduce and control the disease in the greenhouse or field. Moreover, this simple staining technique would be an effective tool for rapid identification of downy mildew–resistant or susceptible basil plants in breeding programs.

CONCLUSIONS

This technique represents the first practical means by which sweet basil growers and researchers can diagnose and control the spread of basil downy mildew (P. belbahrii). The characteristic branching of P. belbahrii hyphae can be clearly identified using less than 10× magnification after using this staining method. A dissecting microscope or even a magnifying glass would provide the necessary equipment for identification purposes. The distinct contrasting image obtained using this rapid staining method allows for quick identification of the pathogen at the earliest stages of infection with a minimal sample preparation. Compared to molecular techniques such as PCR, this protocol describes an easier, less expensive, and more rapid technique that does not require sophisticated equipment to identify presence of the pathogen. This diagnostic capability allows for appropriate disease control measures to be taken as a function of the pathogen life cycle, which itself can be studied using the technique described in this paper. Early detection of the disease allows for control of its spread by allowing for the identification and quarantine or disposal of infected plants or the application of chemical control agents to reduce and control the disease in the greenhouse or field. Moreover, this simple staining technique would be an effective tool for rapid identification of downy mildew–resistant or susceptible basil plants in breeding programs.

LITERATURE CITED

Bartnicki-Garcia, S. 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annual Review of Microbiology 22: 87–108.
Belbahri, L., G. Calmin, J. Pawlowksi, and F. Lefort. 2005. Phylogenetic analysis and real time PCR detection of a presumably undescribed Peronospora species on sweet basil and sage. Mycological Research 109: 1276–1287.
Choi, Y. J., H. D. Shin, and M. Thines. 2009. Two novel Peronospora species are associated with recent reports of downy mildew on sages. Mycological Research 113: 1340–1350.
Diez-Navajas, A. M., C. Greep, A. Poutaraud, and D. Merdinoglu. 2007. Two simplified fluorescent staining techniques to observe infection structures of the oomycete Plasmodiurn viticola in grapevine leaf tissues. Micron (Oxford, England) 38: 680–683.
Farahani-Korofet, R. D., P. Romer, and R. Grosch. 2012. Systemic spread of downy mildew in basil plants and detection of the pathogen in seed and plant samples. Mycological Progress doi:10.1007/s11557-012-0816-z.
Garibaldi, A., M. L. Gullino, and G. Minuto. 1997. Diseases of basil and their management. Plant Disease 81: 124–132.
Garibaldi, A., A. Minuto, G. Minuto, and M. L. Gullino. 2004. First report of downy mildew on basil (Ocimum basilicum) in Italy. Plant Disease 88: 312.
Garibaldi, A., A. Minuto, and M. L. Gullino. 2005. First report of downy mildew caused by Peronospora sp. on basil (Ocimum basilicum) in France. Plant Disease 89: 683.
Garibaldi, A., D. Bertetti, and M. L. Gullino. 2007. Effect of leaf wetness duration and temperature on infection of downy mildew (Peronospora sp.) of basil. Journal of Plant Diseases and Protection 114: 6–8.
Hansford, C. G. 1933. Annual report of the mycologist. Review of Applied Mycology 12: 421–422.
Hoch, H. C., C. D. Galvani, D. H. Szarowski, and J. N. Turner. 2005. Two new fluorescent dyes applicable for visualization of fungal cell walls. Mycologia 97: 580–588.
Jackson, B. P., and D. W. Snowdon. 1990. Atlas of microscopy of medicinal plants, culinary herbs and spices. Bellhaven Press, London, United Kingdom.
Juliani, R., A. Koroch, and J. E. Simon. 2008. Basil: A source of rosmarinic acid. Dietary Supplements ACS Symposium Series, vol. 987, 129–142. American Chemical Society, Washington, D.C., USA.
Khatiri, H., G. Calmin, N. Moarrefzadeh, L. Belbahri, and F. Lefort. 2007. First report of downy mildew caused by Peronospora sp. on basil in northern Iran. Journal of Plant Pathology 89: S70.
McLeod, A., S. Coertzel, and L. Mostert. 2006. First report of a Peronospora species on sweet basil in South Africa. Plant Disease 90: 1115.
Roberts, P. D., R. N. Radd, P. F. Harmon, S. A. Jordan, and A. J. Palmateer. 2009. First report of downy mildew caused by a Peronospora sp. on basil in Florida and the United States. Plant Disease 93: 199.

http://www.bioone.org/loi/apps

3 of 4
RONCO, L., C. ROLLÁN, Y. J. CHOI, AND H. D. SHIN. 2009. Downy mildew of sweet basil (Ocimum basilicum) caused by Peronospora sp. in Argentina. Plant Pathology 58: 395.

SAVORY, E. A., L. L. GRANKE, L. M. QUESADA-OCAMPO, M. VARBANOV, M. K. HAUSHEK, AND B. DAY. 2011. The cucurbit downy mildew pathogen Psuedoperonospora cubensis. Molecular Plant Pathology 12: 217–226.

SIMON, J. E., J. QUINN, AND R. G. MURRAY. 1990. Basil: A source of essential oils. In J. Janick and J. E. Simon (eds.), Advances in new crops, 484–489. Timber Press, Portland, Oregon, USA.

SPENCER, D. M. 1981. The downy mildews. Academic Press, London, United Kingdom.

THINES, M., S. TELLE, S. PLOCH, AND F. RUNGE. 2009. Identity of the downy mildew pathogens of basil, coleus, and sage with implications for quarantine measures. Mycological Research 113: 532–540.

VILLANI, T. S., A. K. KOROCH, AND J. E. SIMON. 2013. An improved clearing and mounting solution to replace chloral hydrate in microscopic applications. Applications in Plant Sciences 1: 1300016. doi:10.3732/apps.1300016.

WICK, R. L., AND N. J. BRAZEE. 2009. First report of downy mildew caused by a Peronospora species on sweet basil (Ocimum basilicum) in Massachusetts. Plant Disease 93: 318.

WILLIAMSON, B., W. A. BRESEE, AND R. C. SHATTOCK. 1995. A histological study of downy mildew (Peronospora rubi) infection of leaves, flowers and developing fruits of Tummelberry and other Rubus spp. Mycological Research 99: 1311–1316.

WYENANDT, C. A., J. E. SIMON, M. T. McGrath, AND D. L. WARD. 2010. Susceptibility of basil cultivars and breeding lines to downy mildew (Peronospora belbahrii). HortScience 45: 1416–1419.