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## An Easy Direct Zoosporangia Sampling Method for Collecting *Phytophthora infestans* Isolates

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**Keywords:** *Phytophthora infestans* • sample collection • isolation • potato late blight

### Abstract

Our new direct zoosporangia sampling (DZS) method was compared with the conventional newspaper bag method as an easy technique for sampling and isolating *Phytophthora infestans*. Field tests were conducted in the Tokachi region, Hokkaido, Japan, in 2016 and 2017. With the new method, zoosporangia on the lower side of diseased leaves in the field were brought into direct contact with selective medium (Rye-B agar with rifampicin, ampicillin sodium and nystatin at concentrations of 25, 100 and 50  $\mu\text{g}/\text{mL}$ , respectively). The collected zoosporangia on the agar were then transported to the laboratory under cool conditions for isolation. With the paper bag method, the diseased leaves collected in the newspaper bags were kept in the laboratory for 0, 1, 2 or 3 days. Then, the zoosporangia on each lesion were transferred to selective medium to isolate *P. infestans*. For the 500 samples collected with the two methods, there was no significant difference in the number of successful isolations. The new DZS method is comparable to the conventional paper bag method. In addition, it is not necessary to perform initial subcultures in the laboratory with the DZS method. Therefore, this simplified method saves much labor and time when collecting isolates of *P. infestans*.

### I. Introduction

Potato late blight caused by *Phytophthora infestans* (Mont.) de Bary results devastating damage to potatoes. Black or brown lesions on leaves appear dark green, water-soaked with chlorotic borders (Fig. 1). Under humid conditions, *P.*

*infestans* produces zoosporangia around the lesion below the surface of the infected tissue. To study the national distribution of this pathogen, collecting many samples from a broader area is necessary to collect accurate data.

The conventional method for sampling *P. infestans* on leaves consists of collecting leaves with lesions in bags made from folded newspapers in the field (Sato et al. 1991) (Fig. 2), transporting the diseased leaves under cool conditions to the laboratory, and isolating fungi by transferring zoosporangia from the lesions to Rye-B agar (Caten and Jinks 1968) with ampicillin sodium, nystatin, and rifampicin. The concentrations of antibiotics were modified slightly from Gareth et al. (1995). With the conventional paper bag method, *P. infestans* is transferred from diseased leaves to selective medium in the laboratory soon after sampling or receiving samples from other collectors. However, this method is too laborious to deal with a large number of samples and there is a possibility that transportation of samples for several days made sample leaflets worse. Therefore, an easier, more labor-saving sampling method is required to collect many samples from the field. Therefore, we examined whether a new method, called the direct zoosporangia sampling (DZS) method, facilitates isolation procedures and increase the isolation rate.

## II. Materials and Methods

Field tests were conducted in the Tokachi region, Hokkaido, Japan, where potato late blight infestations occurred in 2016 and 2017. The DZS method consists of the following steps:

1. *P. infestans* selective medium were made. Autoclaved Rye-B agar (Caten and Jinks 1968) was incubated at 53°C. Rifampicin (code number: 185-01003; Wako Pure Chemical Industries, Osaka, Japan), ampicillin sodium (code number: 016-10373; Wako Pure Chemical Industries) and nystatin (catalog number: N3503-MU; Sigma-Aldrich, St. Louis, MO, USA) were added at concentrations of 25, 100 and 50  $\mu\text{g}/\text{mL}$ , respectively.
2. Selective medium were dispensed into 6-cm Petri dishes (product number: 351007; Corning, NY, USA), which were put in a Styrofoam box with an ice pack to keep the temperature  $< 10^\circ\text{C}$  for transport to the field.
3. In the field, a Petri dish was opened and the surface of the selective medium was gently brought into contact with zoosporangia sporulating on the lesion tissue (Fig. 3a).
4. The Petri dishes were then closed and put into a plastic bag. The plastic bag was placed in a Styrofoam box with ice packs to keep the temperature  $< 10^\circ\text{C}$  and then transported to the laboratory.
5. In the laboratory, the samples were immediately incubated at 15°C in the dark for fungal isolation. Isolated *P. infestans* were preserved on Rye-B agar slants as stocks.

To compare the usability of the DZS and paper bag methods, five leaflets were chosen from each diseased leaf on the same plant or adjoining plants at the same symptom development stage: zoosporangia on one diseased leaflet were sampled using the DZS method and the other four diseased leaflets were collected using the paper bag method. The samples were transported to laboratory within 3-6 h and put in a refrigerator ( $\sim 4^{\circ}\text{C}$ ) on arrival.

With the paper bag test, the collected diseased leaves were kept for 0, 1, 2 or 3 days and then zoosporangia were transferred to selective medium from the leaves. After confirming the presence of zoosporangia on the medium under a microscope, the medium was cultured at  $15^{\circ}\text{C}$  for approximately 10 days. Isolation was scored as successful when there were viable *P. infestans* mycelia on the medium.

For this test, 500 samples (100 with the new method and 400 with the newspaper bag method) were analyzed (Table 1). To compare the DZS and paper bag methods, the number of isolates on 0, 1, 2 and 3 days with the conventional method were analyzed using the chi-square test ( $\chi^2$ test) with SPSS ver. 22 (IBM, Armonk, NY, USA).

### III. Results

With the DZS method, it was easier to sample *P. infestans* in the field by collecting zoosporangia directly on the medium. After culturing for a few days, the adhering zoosporangia germinated and developed mycelia (Fig. 3b-d).

There were 26 successes in 2016 and 47 successes in 2017 with the DZS method, and 18 to 25 successes in 2016 and 50 to 56 successes in 2017 with the paper bag method (Table 1). Although the success rate in 2016 was lower than in 2017 for both methods, the number of successes was high (68-81/100 samples) regardless of the keeping days or isolation methods, for which no significant difference was found.

**Table 1.** Successful isolation with the direct zoosporangia sampling (DZS) and paper bag methods

		DZS method	Paper bag method (days)			
			0	1	2	3
2016 (n = 39)	Success	26	25	25	20	18
	Failure	13	14	14	19	21
2017 (n = 61)	Success	47	53	56	50	50
	Failure	14	8	5	11	11

2016, 2017 and the total number of successes were analyzed and there was no significant differences by the  $\chi^2$  test ( $P > 0.05$ )

#### IV. Discussion

The DZS method appears to be an easy new method for sampling *P. infestans*. Using the DZS method, isolation was successful in 73/100 samples in field tests. Although there was no difference in the success rate between the DZS and paper bag methods, there were more cases with no viable *P. infestans* isolate 2 days after using the paper bag method than with the new method. It takes about 3 days to receive samples collected with the conventional paper bag method by mail from other collectors. Considering this, the DZS method is useful compared with the conventional paper bag method.

Failures of isolation due to contamination were more frequent with the DZS method than with the paper bag method, possibly because microorganism activity may be kept under the conditions used for the DZS method. However, even if there is bacterial or fungal contamination, it is easy to distinguish and eliminate these contaminants from *P. infestans* by identifying macroscopic characters or the microscopic shape of the spores.

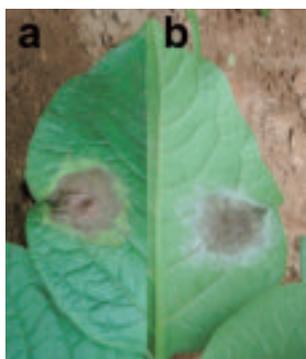
The DZS method facilitates isolation by eliminating the time required to transfer zoosporangia from diseased leaves to selective medium. The conventional paper bag method needs to transfer zoosporangia from collected diseased leaves to selective medium in the laboratory, which takes about 1 hour for every 10 samples. In comparison, the DZS method simply puts the *P. infestans* on selective medium in the field; therefore, it saves time and labor when dealing with a large number of samples. In the DZS method, it is possible to increase the isolation rate by subculture of isolates under suitable conditions with precise manipulation.

#### Acknowledgments

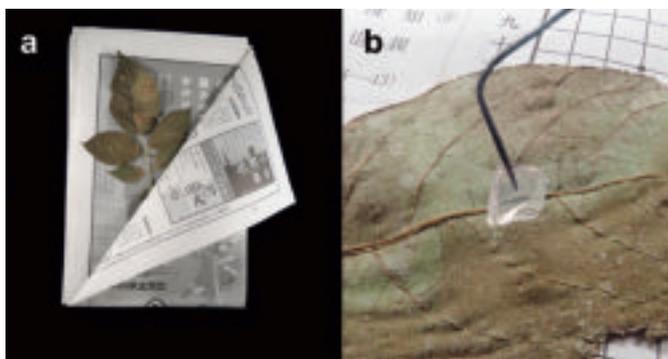
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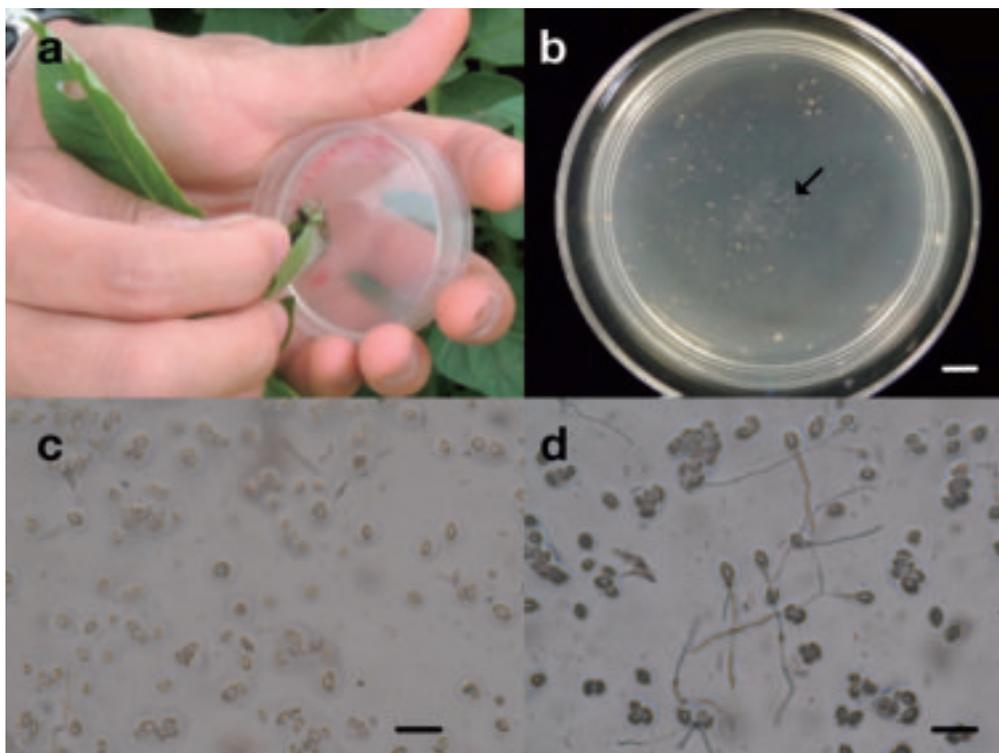
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**Fig. 1.** **a** A photograph of the single lesion of potato late blight on upper side of leaf. **b** A photograph of the single lesion of potato late blight on the lower side of leaf.



**Fig. 2.** **a** Diseased leaflet sample in the newspaper bag. **b** Picking up zoosporangia by agar tip for transfer to selective medium



**Fig. 3.** **a** A photograph of the direct zoosporangia sampling (DZS) method in the field. To sample zoosporangia, a lesion is gently brought into contact with the selective medium. **b** Zoosporangia on the selective medium after sampling; there are many zoosporangia near the arrow. **c** Zoosporangia on the medium viewed by microscopy from the back side of the Petri dish. **d** Germinating zoosporangia on the medium. Scale bar in **b** 1 cm, in **c** and **d** 100  $\mu\text{m}$