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A Simple Method for Estimating Conidial Numbers of Japanese Pear Scab in Suspension

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Abstract

A proposal is made for efficiently estimating conidial numbers of Japanese pear scab in rain water. The proposed concentration method consists of a few steps as follows; conidia are trapped in an agar solution by adding a small amount of diluted agar solution to conidial suspension, centrifuging them, and pouring supernatant water out. It was confirmed that seasonal changes in the number of conidia in rain water which flows down on a Japanese pear shoot were pursued with this method effectively and efficiently. Through this method, changes in the number of conidia at the primary infection source, formed on scale lesions were, also identified: i.e. scale lesions appeared in late January and bud lesions did before flowering.

Discipline: Plant disease
Additional key words: agar solution concentration (ASC) method, primary infection source, *Venturia nashicola*

Introduction

Establishment of a method for effectively detecting a causal pathogen is very important for ecological studies on plant diseases, including Japanese pear scab (*Venturia nashicola*). For the purpose of concentration, it is usual to centrifuge conidial suspensions[4,11]. Japanese pear scab conidia, however, tend to float on the suspension surface, and centrifugation efficiency falls short of expectations. The author conceived an idea of establishing a simple method with high concentration efficiencies, adding a small amount of agar solution to the suspensions before centrifuging. It was confirmed that the proposed method was suitable for practical usage[13].

The present paper attempts to review the agar solution concentration (ASC) method developed, and its applicability to ecological studies on Japanese pear scab, especially those on its primary infection[13,14].

Materials and methods

1) Coagulation of agar solution

Agar powder was added to deionized water at rates of 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5% by weight, and was dissolved completely in an about 90°C water bath. The agar solutions were each divided into two parts, one of which was kept at room temperature of 20°C and the other refrigerated to 5°C for coagulation tests.

2) Amounts of agar solutions deposited after centrifugation

10 m*l* samples of conidial suspensions of Japanese pear scab were placed in a centrifugation tube of 10 m*l* capacity and centrifuged after separately adding equal amounts of pure agar, i.e. 1 m*l* of 0.05% agar solution, 0.5 m*l* of 0.1% solution and 0.25 m*l* of 0.2% solution, respectively. Supernatant water was poured out after centrifuging at 650 × G for 5 min.

3) Counting numbers of conidia

A completely mixed sample of agar solution remained at the bottom of the centrifugation tube after centrifuging was investigated with a hemacytometer over 5 times under a microscope. Investigated part in the hemacytometer was a slit portion, and a

parallel portion around the slit in cases where very few conidia were observed. The volume in the latter case was 3.47 times as compared with the portion of only slit. Actual numbers of the spores were therefore estimated from those investigated number divided by 3.47 in that case. The suspension of 0.2 m*l* sampled from the upper and bottom portions in the centrifugation tube after cenrifuging without an addition of agar solution was investigated on numbers of conidia with the same manner as the case of adding agar solution.

4) Preservation of agar solution

The agar solution was preserved in a refrigerator at about 5°C with or without p-hydroxybenzoic acid 2 g and sorbic acid 1 g per 1 *l* of agar solution.

5) Breakup of conidia in water

The Eyer-spreader (the commercial name is Eyer, the main components of which are polyoxyethylen alkylphenylether 10% and polyoxyethylen fatty-acidester 10%), 50-times diluted, was added to collected rain water at a rate of 1% to water volume, and the suspension was fully shaken.

6) Collection of rain water from pear tree shoots

Cultivars Hosui and Chojuro cultivated with trellis training were used for the present study. Rain water was collected at the basal point of the shoots which were kept at angles of about 40 to 60 degrees upward from the horizontal line. These shoots were tightly wrapped with an aluminium foil which made a funnel to collect rain water and store it in a polyethylene 3 *l* tank kept on the ground and connected by a vinyl pipe. Mouth of the tank was covered with an aluminium foil to prevent the invasion of rain water and dust. Collections of rain water were continued during the period of early September to mid November. The samples were taken at 9:00 a.m. after each rainfall.

7) Numbers of conidia in rain water

The amount of rain water was measured for each shoot from which the water was collected. An amount of 1 m*l* of the Eyer-spreader, 50-times diluted, was added to every 100 m*l* of water for the purpose of facilitating conidial breakup before centrifugation, provided that more than 10 m*l* of rain water was collected. When the amount of collected rain water was less than 10 m*l*, deionized water was added to reach 10 m*l*, which was centrifugated after adding a small amount of agar solution. The number of conidia was counted with a hemacytometer under a microscope. Investigations in the experiment were made into the portions 3.47 times in volume as large as a slit portion by including a parallel portion. A total number of conidia was estimated by the following equations:

In cases where more than 10 m*l* of rain water was collected,

$$C = W \cdot N \cdot 10^4 / 3.47 \quad W \cdot N \cdot 101/100, \text{ and}$$

in cases where less than 10 m*l* of rain water was collected,

$$C = W \cdot N \cdot 10^4 / 3.47 \cdot 10/W,$$

where, C: total estimated number of conidia, W: volume of collected rain water, and N: average conidial number under microscopic investigations.

8) Number of conidia formed on scale lesions

Diseased and healthy flower-bud scales were sampled during the period February 8 to April 19, 1989 at intervals of 7 to 22 days. Each of them was thoroughly rinsed with 10 m*l* of deionized water and concentrated by centrifuging after adding 0.5 m*l* of 0.1% agar solution.

9) Germinability of spores

Conidial germinability was tested on water agar (WA) in both petri dishes and on glass slides. Germination of each conidia was evaluated according to Mizusawa's criteria[6], under which the term of "germination" was employed when germ-tube length reached more than one half of its short dimension after being kept in the dark at 15°C for 48 hr.

Results

1) Coagulation of agar solution

The agar solutions of 0.05 and 0.1% did not coagulate at 20°C and 5°C, while the 0.2% solution coagulated at 5°C and the solutions of more than 0.3% also did at even 20°C. These results suggest that a 0.1% agar solution be the most suitable for this study from the viewpoint of interactions between coagulation temperatures and concentration of agar solution.

2) Relationship between centrifugation period and deposited agar solution

Almost no agar solution was deposited when the conidial suspension containing 0.5 m*l* of 0.1% agar solution was centrifuged for 1 min. However, agar solution was deposited at the bottom of the centrifugation tube when the suspension was centrifuged for more than 5 min, and the amount of deposited agar solution was constant irrespective of a centrifugation period. The number of conidia caught in the agar solution did not change during the centrifugation for 5 to 60 min (Table 1).

Table 1. Number of conidia in agar solution after different periods of centrifugation[a)]

Time (min)	Number of conidia[b)] $\bar{x} \pm$ S.D.
1	—[c)]
5	81.3 ± 26.2
10	87.0 ± 42.1
20	83.0 ± 29.4
60	80.7 ± 21.0

a): 1 m*l* of 0.1% agar solutiion was added to 10 m*l* of the original suspension having 4.0 conidia per 10^{-3} m*l*, and 3.6 of S.D.
b): Number of conidia per 10^{-3}m*l* (mean of 30 trials each).
c): Not coagulated.

3) Relationship between concentration of added agar solution and deposited agar solution

Amounts of the deposited agar solution at the bottom of the centrifugation tubes, each containing 10 m*l* deionized water and an equal amount of pure agar solution, i.e. 1.0 m*l* of 0.05%, 0.5 m*l* of 0.1%, or 0.25 m*l* of 0.2% solution, were examined after centrifuging. The amounts of the deposited agar solution were almost the same, i.e. 0.2 m*l*, in each case. The numbers of conidia in agar solutions also did not show significant variation (Table 2).

Table 2. Number of conidia in agar solution having different combinations of concentration and volume after centrifugation[a)]

Concentration of agar solution (%)	Volume added (m*l*)	Centrifugation	Volume of agar solution[b)] (m*l*)	Number of conidia[c)] $\bar{x} \pm$ S.D.
0	0	−	−	4.3 ± 3.2
0.05	1.0	+	0.2	120.2 ± 22.8
0.1	0.5	+	0.2	124.5 ± 29.4
0.2	0.25	+	0.2	134.3 ± 42.4

a): Volume of the original conidial suspension; 10 m*l*.
b): At a bottom of tube after centrifugation.
c): Number of conidia per 10^{-3} m*l* (mean of 12 trials each).

Table 3. Effects of antiseptic and spreader on germination of conidia and elongation of germ tube on a glass slide

Addition of antiseptic[a)]	Concentration of spreader added (%)	Germination		Germ tube	
		Number of conidia counted	Rate[b)] (%)	Number of conidia measured	Length $\bar{x} \pm$ S.D. (μm)
+	0.02	336	67.6 c	25	63.8 ± 25.5
−	0.02	365	53.7 d	25	55.5 ± 23.8
+	0.01	344	72.7 bc	25	68.8 ± 28.3
−	0.01	390	76.2 ab	25	65.5 ± 28.0
+	0	359	83.8 a	25	67.0 ± 30.5
−	0	319	76.5 ab	25	85.0 ± 23.3

a): +; added, −; not added.
b): Figures in each column followed by the same letters are not significantly different $P \leqq 0.05$ (Duncan's multiple range test).

Table 4. Number of conidia in different portions of suspension in tube after centrifugation

Treatment of conidial suspension			Portions of sample in tube[c]	Number of conidia[d] $\bar{x} \pm$ S.D.
Centrifugation[a]	Addition of agar solution[b]	Re-centrifugation of supernatant[a]		
−	−	−	Whole	2.94 ± 1.47
+	−	−	Upper	0.72 ± 0.81
+	−	−	Bottom	3.75 ± 2.97
+	+	−	Upper	0.09 ± 0.17
+	+	−	Bottom	112.10 ± 47.87
+	+	+	Bottom	1.93 ± 0.95

a): 25 min at $650 \times g$ (+), not centrifuged (−).
b): 0.5 m/ of 0.1% agar solution was added (+), not added(−).
c): Bottom; portion of agar solution.
d): Number of conidia per 10^{-3} m/ (mean of 20 trials each).

Table 5. Effect of volume of agar solution added to the suspensions on their concentration efficiency after centrifugation

Agar solution added[a] (m/)	Number of conidial suspensions	Concentration efficiency[b] $\bar{x} \pm$ S.D.
0.5	5	41.0[c] ± 6.16
1.0	9	22.1 ± 5.10

a): Agar concentration is 0.1%.
b): Ratio of number of conidia per m/ of agar solution after centrifugation to that of the original suspension (Mean of 10 to 50 trials each).
c): Average of 5 or 9 suspensions.

4) Prevention of decomposition of agar solution

Effects of the two antiseptics preventing decomposition of agar solution on ratios of germination and elongation of germ-tube were investigated on WA and a glass slide. The ratio of germination was slightly low in some cases of the glass slide test, while no difference was recognized in the WA test. No significant differences were observed in germ-tube length among the treatments, and amounts of the deposited agar solution were also constant among them (Table 3).

5) Location of conidia in a centrifugation tube after centrifuging

When a small amount of agar solution was added to suspensions, a great number of conidia were observed in the deposited agar solution located at the bottom of the centrifugation tube. Conidia were rare in supernatant water after centrifuging the suspensions, and a very few conidia were recognized in the agar solution after re-centrifuging the supernatant water. However, when no agar solution was added, only some conidia existed in the water sampled from the bottom and a few conidia were observed in the samples from the upper portion after centrifuging (Table 4).

6) Concentration efficiency

The concentration efficiency was evaluated by comparing the average number of conidia in the original suspension and agar solution after centrifuging. The concentration efficiency was ranged from 33.3 to 49.2, averaging 41.0 ± 5.1 when adding 0.5 m/ of 0.1% agar solution, while it was 12.0 to 24.9, averaging 22.1 ± 5.1 when adding 1.0 m/ of 0.1% agar solution. A theoretical value of concentration efficiency is calculated by dividing the volume of original suspension by the volume of the deposited agar solution, and the loss in process of centrifugation is a difference between the theoretical value and the experimental one. The loss in the case of adding 0.5 m/ of 0.1% agar solution was 18%, i.e. $(1-41/50) \times 100$, which was a little smaller than the theoretical value of 50. In the case where 1.0 m/

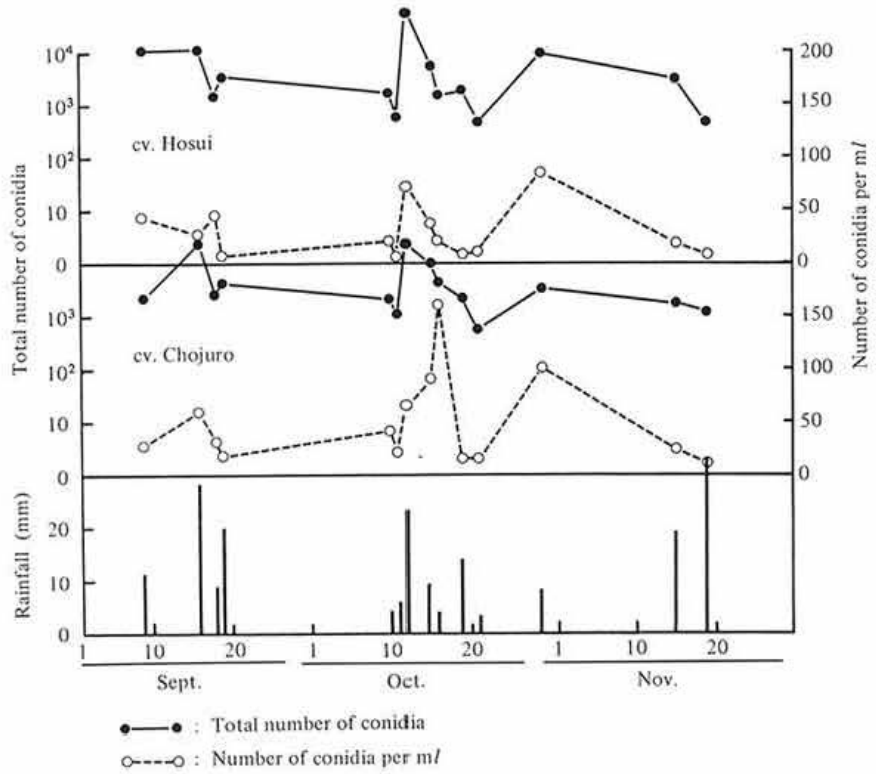

Fig. 1. Number of conidia in rain water flowering down on a shoot

of 0.1% agar solution was added, the loss was 11.6%, i.e. $(1-22.1/25)\times 100$, while the theoretical value was 25 (Table 5).

7) Numbers of conidia in rain water collected from Japanese pear tree shoots

The presence of conidia in rain water was observed from the start of rain water collection in early September through the pear leaves fall season. The number of conidia per 1 m*l* of collected rain water varied from 22 to 543 in 1983, and from 7 to 160 in 1984. Total numbers of conidia in the rain water collected from one shoot ranged from 209 to 335,140 per day in 1983, and from 442 to 53,214 per day in 1984. These variations in the number of conidia seemed to be more closely related to the number of days of water collection from the beginning of rainfall and its total amount of collected rain water, rather than to the time of collection. The total number of conidia in the collected rain water was greater in cv. Chojuro than in cv. Hosui (Fig. 1).

8) Numbers of conidia formed on scale lesions

The total number of conidia formed on scale lesions varied from 190 to 1,137 per bud on 8 February 1990. It was very likely that some old conidia produced on other part of lesions last year were also included. The total number of conidia varied from 228 to 758 per diseased bud during the period 2 to 23 March, followed by a rapid increase after 4 April. One of the noticeable points presented in Table 6 is that the number of conidia collected from healthy buds decreased in accordance with delayed collection, reaching zero after March 23.

9) Ratio of conidial germination formed on scale lesions

The ratio of conidial germination collected from bud scales was approximately 30% on 8 February, possibly including old survival conidia formed last year, while it was generally over 70% after 2 March. These results seem to indicate an increase in newly formed and highly germinable conidia (Table 6).

Table 6. Changes in number of conidia and their germinability

Sampling date	Scale	No. of buds investigated	No. of conidia per bud	Ratio of conidial germination
Feb. 8	Diseased	1	1,137	26.8
	Healthy	2	190–442	25.0–31.3
Mar. 2	Diseased	1	758	83.3
	Healthy	3	0–316	0–14.3
Mar. 13	Diseased	1	758	96.3
	Healthy	2	0–63	0
Mar. 23	Diseased	2	228–253	60–73.9
	Healthy	2	0	0
Apr. 4	Diseased	3	126–4,486	40–95.1
	Healthy	2	0	0
Apr. 12	Diseased	3	1,137–8,340	63.3–90.9
	Healthy	2	0	0
Apr. 19	Diseased	1	442–8,593	82.7–100
	Healthy	2	0	0

Discussion

In estimating the numbers of conidia of Japanese pear scab in conidial suspension, some steps are required as follows: conidial suspension is centrifuged for a certain period of time, supernatant water is then poured out, and the number of conidia in water at the bottom is counted under the microscope with a hemacytometer[4,11]. However, this method is apparently not highly effective, because Japanese pear scab conidia tend to float in water. The critical improvement in the new concentration method as proposed above is to add a small amount of agar solution to the conidial suspension. In this way conidia can be effectively trapped in the solution. The agar solution does not decompose without antiseptics for 6 to 7 months if it is preserved in a refrigerator.

Uniform dispersion of conidia in water is essential to counting precisely the number of conidia in collected rain water after centrifuging part of it. Toward this end, the Eyer-spreader, 50-times diluted, was added to rain water at the rate of 1% by volume and fully shaken. None of the tests under the present study showed any adverse effects on conidial germination and germ-tube growth, resulting from adding spreader. It is therefore concluded that conidia obtained through this method can be available for inoculation experiments, in which conidial germination has an important implication.

Concentration efficiency of the proposed method depends upon three factors: i.e. conidia density, amount of added agar solution, and capacity of a centrifugation tube. If the centrifugation tube has a larger size, for example 10 times in volume, the concentration efficiency is increased by 10 times. However, it is also possible to increase concentration efficiency by reducing the amount of added agar solution. The recommended combination for the proposed method is: the capacity of the centrifugation tube is 10 m/ and the added agar solution is 0.5 m/ of 0.1%, both of which are subject to degree of dust contamination in agar solution and amount of the deposited agar solution for investigation.

The proposed method could be applicable to concentrating the suspension of water-borne spores of other pathogens such as grapevine swelling arm[9] and causal fungi of *Phomopsos* sp.[2]. The proposed method is called "agar solution concentration method" with an abbreviation of ASC method[13].

It is presumed that an infection source of Japanese pear scab fungus to scales of buds on shoots is conidia contained in rain water, which flows down along the pear shoots in autumn, since a considerable number of conidia are observed in the collected rain water. These conidia may be originated from autumn scab lesions[12], which usually take place on the lower parts of leaf surfaces in early autumn, while the germinability of conidia which are produced on spring scab lesions[12] is rather low in autumn. It is confirmed that apple scab fungi overwinter in a state of conidia on the lesions in infected scales as

a primary infection source[1,3,7,8,10]. The present study also confirmed that Japanese pear scab fungi overwinter on infected scales of buds on shoots and form conidia in the later part of winter season[4,5,14]. In regards to disease development in scales, a lesion appears in late January, conidiophores are formed in early February, conidia have high germinability in early March, after which the infection by conidia gradually increases[14].

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