Effect of Culture Conditions on Conidia Formation by *Elsinoë ampelina*, the Causal Organism of Grapevine Anthracnose

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ABSTRACT

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Elsinoë ampelina, the causal organism of grapevine anthracnose, can be easily grown in culture, yet its sporulation is poor and unstable in culture. In this study, we sought the optimum conditions for a simple method to stably generate conidia. We first examined the optimum period of incubation for young colonies grown on potato dextrose agar (PDA) in water. The resultant number of conidia showed a logarithmic increase, which slowed at about 8 to 10 h. This suggests that 8 to 10 h of preculture would provide a sufficient number of conidia under the culture conditions used. A high negative correlation between colony density on PDA and the number of resultant conidia existed: colonies grown at >2.5 colonies per cm² produced few or no conidia, whereas those grown at <1.0 colony per cm² stably produced as many as 2.9×10^6 conidia. The optimum condition for preculture was to incubate colonies grown for 6 days on PDA at a density of <1.0 colony per cm². The conidia obtained by our method were pathogenic on the grape cultivar Rizamat.

Grapevine anthracnose is a disease of European origin that reduces the quality and quantity of the crop (9). *Vitis vinifera*, the European species of grapevine, is susceptible to anthracnose, whereas some American grapevine species, such as *V. labrusca* and *V. aestivalis*, are good genetic resources of resistance to this disease (3,4,10). Because anthracnose is transmitted by rain-splash, the damage is severe in rainy and humid regions, including Japan. Therefore, frequent fungicide applications are indispensable for the control of this disease in Japanese viticulture.

Anthracnose lesions are circular and have the appearances of "shot-holes" (8,9).

The causal organism of this disease is *Elsinoë ampelina* (de Bary) Shear, which produces small, ovoid, hyaline conidia (3–6 × 2–8 µm) with mucilaginous walls and one or two refringent spots (9,12,14). Other species of *Elsinoë* cause anthracnose disease on raspberry (*E. veneta*) and scab diseases on citrus (*E. fawcettii* and *E. australis*) and on avocado (*E. perseae*) (1).

E. ampelina is able to grow slowly and form compact colonies on artificial media, although it rarely sporulates under artificial conditions (10,15). The difficulty in preparing conidial suspensions from pure culture makes it challenging to evaluate the resistance of grapevine cultivars to anthracnose. Timmer et al. (16) reported that conidia of *E. fawcettii*, the causal organism of citrus scab, could be obtained by irradiating mycelia on artificial media with long-wavelength UV light. Almost the same method using near-UV irradiation was used to obtain conidia of *E. ampelina* (17). Hopkins and Harris (6) obtained conidia of *E. ampelina* by flooding sterile water onto a 3- to 4-week-old culture. These methods are not convenient, however, because in the former it is difficult to achieve the precise condition for UV irradiation, and the latter requires an inconveniently long period of incubation. In this study, we investigated the effects of culture conditions on formation of *E. ampelina* conidia and developed a reliable, simple, and rapid method for the preparation of conidia for inoculation tests.

MATERIALS AND METHODS

Isolation and maintenance of *E. ampelina*. Isolate Akitsu01 of *E. ampelina* was collected from naturally infected leaves of 'Kyoho' grown in the Department of Grape and Persimmon Research, National Institute of Fruit Tree Science, Japan. The fungus was grown on potato dextrose agar (PDA) plates, composed of 24 g/liter potato dextrose broth (Difco, Becton, Dickinson and Company, NJ, USA) and 2% (wt/vol) agar (Wako, Osaka, Japan), at 24°C in the dark. Conidial suspension in water containing 20% (vol/vol) glycerol was stored at –80°C.

Culture conditions. Small amounts of conidia scraped from the surface of full-grown reddish colonies or obtained by the method described below were inoculated onto 9-cm-diameter PDA plates and kept at 24°C in the dark as a preculture. To prepare the conidial suspension, approximately 10 colonies from preculture plates were incubated in 200 µl of sterile water in a 2-ml tube either with shaking (150 revolutions min^{–1}) or without, in the dark, at 24°C following the methods of Kore and Gurme (7).

Optimum period of culture. Colonies were incubated in the dark at 24°C for 5 or 6 days on PDA plates in water as described above. Eight microliters of the supernatant were sampled after 0, 3, 6, 8, 10, and 24 h or after 0, 2, 4, 6, 8, 10, and 24 h to measure the number of conidia produced, which was calculated from the concentration in the supernatant measured with an improved Neubauer hemacytometer. The concentration of conidia was determined by counting in four 1-mm² partitions of the hemacytometer and calculating the mean and standard error (SE) of conidia per partition. Experiments were repeated 3 times with 2 replicates (6 replicates in total).

Effect of colony density on conidial production. Various concentrations of conidial suspension were inoculated onto PDA plates and the number of resultant colonies on each plate counted. Colony density was calculated by dividing the number of colonies by the plate area. Colonies were sampled for 1 production of conidia from 25 PDA plates (6 repeats, 3–5 replicates) after 4 days of incubation, 33 plates (8 repeats, 3–6 replicates) after 5 days, and 28 plates (6 repeats, 3–5 replicates) after 6 days. The number of conidia

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produced was calculated from the concentration in the supernatant of the liquid culture as described above.

Effect of preculture period on conidial production. Inoculated plates kept at 24°C in the dark were sampled daily from 4 to 9 days after inoculation. Colonies precultured at >2.5 colonies per cm² were sampled from 8 PDA plates (3 repeats, 2 or 3 replicates) after 4 days of incubation, 15 plates (4 repeats, 3 or 4 replicates) after 5 days, 8 plates (3 repeats, 2 or 3 replicates) after 6 days, and 10 plates (4 repeats, 2 or 3 replicates) after 7 days. Colonies precultured at 1.0 to 2.5 colonies per cm² were sampled from 11 PDA plates (4 repeats, 2 or 3 replicates) after 4 days of incubation, 5 plates (3 repeats, 1 or 2 replicates) after 5 days, 11 plates (4 repeats, 2 or 3 replicates) after 6 days, and 7 plates (3 repeats, 2 or 3 replicates) after 7 days. Colonies precultured at <1.0 colony per cm² were sampled from 8 PDA plates (3 repeats, 2 or 3 replicates) after 4 days of incubation, 17 plates (4 repeats, 3–5 replicates) after 5 days, 11 plates (3 repeats, 3 or 4 replicates) after 6 days, 6 plates (3 repeats, 2 replicates) after 7 days, 8 plates (2 repeats, 4 replicates) after 8 days, and 8 plates (2 repeats, 4 replicates) after 9 days. The number of conidia produced from each sampled plate was calculated as described above. The mean number of conidia produced in each preculture period was calculated for each series of plates, and the SE was calculated from standard deviation and the number of sampled plates.

Statistical analysis. To test whether the number of conidia produced during each preculture period differed significantly by colony density (<1.0, 1.0 to 2.5, or >2.5 colonies per cm²), a Wilcoxon rank-sum test with Holm-adjusted *P* value was used for the multiple comparison.

Inoculation of conidial suspension onto young grapevine leaves. Because rapidly growing leaves are susceptible to anthracnose (2), we used healthy expand-

ing leaves of 'Rizamat' to test the pathogenicity of the prepared conidial suspensions. Sampled leaf blades were 3 to 6 cm in length. Leaves that were intermediate in size between the youngest full-grown leaf and the next younger leaf were selected to sample rapidly growing leaves. Detached leaves were incubated in 0.5% (wt/vol) sterile agar plates with the petioles embedded in the agar. A 1-cm² piece of gauze was placed on a leaf, and 25 µl of conidial 5×10^3 conidia per ml suspension was applied to the gauze. Covered plates were wrapped with polyethylene bags (0.03 mm thick) to maintain >95% relative humidity and incubated at 24°C under light. Gauze and polyethylene bags were removed after 2 days, and the leaves were further incubated under the same conditions. The lids of the petri dishes were kept closed to prevent the leaves from withering. Two to three leaves were used for each inoculation test, and the experiment was repeated six times (14 leaves in total).

RESULTS

Effect of incubation conditions on conidial production. Colonies grown for 5 to 6 days on PDA at a density of <1.0 colony per cm² were incubated in sterile water with or without shaking. Colonies in shake culture produced 2.0×10^6 to 2.5×10^6 conidia (*n* = 5), whereas those in static culture produced 0 to 5.5×10^3 conidia (*n* = 5). Thus, shaking is necessary for the optimum production of *E. ampelina* conidia when they are incubated in water.

The optimum period of incubation in water for the production of conidia was determined. No conidia were produced before incubation in water, whereas newly generated conidia were observed after 3 h of incubation of colonies (Fig. 1). Thereafter, production of conidia increased logarithmically until 8 h of incubation. After 8 h, the increase in the number produced gradually slowed, representing a sigmoidal increase of conidial production (Fig. 1).

Fig. 1. Production of conidia by 10 sampled colonies on six plates of *Elsinoë ampelina* of various colony densities during incubation in distilled water. Vertical bars represent SE (*n* = 4).

Although nearly the same pattern of conidial production was observed from each preculture, the total number of conidia produced from the colonies grown at low density was significantly greater than the number produced from those grown at high density. No conidia germinated before 10 h of incubation, and 0 to 23% of conidia (mean, 7.9%, *n* = 5) germinated after 24 h of incubation.

Effect of preculture conditions on conidial production. Colonies grown at low density on PDA produced more conidia than those grown at high density (Fig. 1). The effect of colony density on conidial production was studied. Because colonies grown for less than 3 days were too small to see, we used colonies grown for at least 4 days. Colonies preincubated for 4 days produced relatively few conidia (Fig. 2A). However, sufficient numbers of conidia were reliably obtained from colonies of both 5- and 6-day incubation at low density, whereas colonies grown at high density produced few or no conidia (Fig. 2B and C). Yields from colonies grown at low density (<2.5 colonies per cm²) ranged from 4.0×10^5 to 2.9×10^6 conidia.

The relationship between the period of preculture and conidial production was also investigated. Because it is difficult to control the precise density of colonies, tested colonies were categorized into three groups: >2.5, 1.0 to 2.5, and <1.0 colony per cm². Colonies precultured at >2.5 colonies per cm² produced fewer than 1×10^5 conidia, on average, at all preculture periods tested, and the number of conidia produced was significantly less than those of other groups for all preculture periods (Fig. 3). Colonies precultured at 1.0 to 2.5 colonies per cm² produced 1.4×10^5 conidia when precultured for 4 days, 6.8×10^5 for 5 days, and 3.6×10^5 for 6 days. However, the colonies precultured for 7 days produced almost no conidia. The number of conidia produced from the colonies precultured for 4 to 6 days at 1.0 to 2.5 colonies per cm² was significantly greater than those from colonies precultured for 4 to 6 days at >2.5 colonies per cm². Surprisingly, many more conidia were produced from the colonies precultured at a density of <1.0 colony per cm²: 1.3×10^5 conidia from colonies precultured for 4 days, 1.0×10^6 for 5 days, 1.7×10^6 for 6 days, 7.0×10^5 for 7 days, and 3.5×10^5 for 8 days. Colonies from 9-day preculture produced only 1.0×10^5 conidia, suggesting that they were losing the ability to produce large numbers of conidia. For 4 to 7 days of preculture, the number of conidia produced from the colonies precultured at <1.0 colony per cm² was significantly greater than those from colonies precultured at >2.5 colonies per cm², and for 6 days of preculture the number was significantly greater than those at 1.0 to 2.5 colonies per cm².

Pathogenicity of resultant conidia. Resultant conidia were inoculated onto young leaves of 'Rizamat'. Typical necrotic lesions (Fig. 4) of anthracnose were created on all 14 inoculated leaves tested, indicating that the conidia obtained by our method maintained their pathogenicity to grapevine.

DISCUSSION

The present study was conducted to develop a simple and time-saving method for obtaining conidial suspensions of *E. ampelina*. First, we determined that colonies incubated in water with shaking could actively produce conidia for up to 8 to 10 h, and then they gradually slowed production, suggesting that a sufficient number of conidia was generated within 10 h. Incubation in water for more than 24 h promoted the germination of conidia. Some phytopathogenic fungi, such as *Alternaria alternata* (5) and *Cochliobolus (Helminthosporium) victoriae* (11), release toxic compounds during conidial germination, and the toxin plays an important role in the early steps of infection (5). Thus, we conclude that conidial suspension including germinated conidia would not be a suitable inoculum. Because freshly produced conidial suspension obtained from 8- to 10-h culture does not include germinated conidia, this would be appropriate for inoculation tests. Therefore, we studied the ability of *E. ampelina* to produce conidia by using colonies from 8-h culture. In addition, a strong negative correlation between the density of colonies in the preculture and the number of produced conidia was observed. The reason for this colony-density-dependent production of conidia is not clear, and its elucidation will require more detailed work. A sufficient number of conidia was easily obtained from colonies precultured on the solid medium for a relatively short period, within 9 days. This finding demonstrates that young colonies can actively produce conidia, and the appropriate period of preculture is relatively short.

To confirm the pathogenicity of conidia obtained by 8-h incubation, conidial suspensions were used as inoculum on young leaves of 'Rizamat'. The typical necrotic lesions formed, and leaf curling, a typical symptom of anthracnose (13), was observed. Thus, inoculated conidia were pathogenic to grapevine and caused anthracnose.

The present study revealed the following three indispensable conditions for the simple and rapid production of conidial suspension of *E. ampelina*: (i) the density of colonies in preculture should be <2.5 colonies per cm²; (ii) depending on colony density, colonies should grow for 4 to 9 days on PDA; and (iii) grown colonies should be cultured in water with shaking for 8 to 10 h. The optimum condition for the preparation of a conidial suspension is

Fig. 2. Correlation between *Elsinoë ampelina* colony density and number of conidia produced. Colonies were grown on potato dextrose agar for **A**, 4 days, **B**, 5 days, or **C**, 6 days, and both colony density and number of resultant conidia obtained by incubation in water were measured. Dotted lines indicate a colony density of 2.5 colonies per cm². Vertical bars represent SE ($n = 4$).

Fig. 3. Relationship between period of preculture of *Elsinoë ampelina* and number of conidia produced. Colonies grown on potato dextrose agar at three densities were cultured in water, and the number of conidia produced was measured. Colonies grown at both >2.5 and 1.0 to 2.5 colonies per cm² were precultured for 4 to 7 days, while those grown at 1.0 colony per cm² were precultured for 4 to 9 days. Average number of conidia produced is shown. Vertical bars represent SE ($n = 5$ to 17). Means with different letters are significantly different ($P < 0.05$) according to Wilcoxon rank-sum test.

Fig. 4. Lesions formed after inoculation of *Elsinoë ampelina* conidia produced by our method. Conidial suspension (25 μ l, 5×10^3 conidia per ml) was inoculated onto the adaxial side of a young leaf of *Vitis vinifera* 'Rizamat'. Photograph was taken 10 days after inoculation. Bar = 5 mm.

to incubate the colonies for 6 days on PDA at <1.0 colony per cm², or for 5 days at 1.0 to 2.5 colonies per cm². The method of Hopkins and Harris (6) required 3 to 4

weeks to culture, and another method using near-UV irradiation (17) requires 12 days of culture in total. Our method requires much less time to obtain a sufficient

number of conidia under simple culture conditions, and only an incubator for the preculture and a shaker for the subsequent culture in water.

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