Enhancement of In Vitro Conidia Production in *Monilinia vaccinii-corymbosi* (Reade) Honey by Cellulose Acetate Membranes

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Abstract. In vitro conidia production by *Monilinia vaccinii-corymbosi* (Reade) Honey, the cause of mummy berry disease in blueberry, was significantly enhanced by cellulose acetate membranes placed on the surface of V-8 juice agar for most of the pathogen isolates tested, compared to V-8 juice agar alone. Temperature and light affected conidia production, but the effects were not consistent. Higher temperature (22 vs. 15 °C) yielded better sporulation, but the effects of light environment were variable. When 55 isolates from various sources were rated visually for sporulation on cellulose acetate membranes at 22 °C under ambient light/dark cycles, a wide range of conidium production was observed, and three of 55 isolates (6%) were identified as having very high conidia production.

Mummy berry, caused by *Monilinia vaccinii-corymbosi*, is an important disease of wild and cultivated blueberries (*Vaccinium* sp.) in the United States and Canada (Eck, 1988). Control of this disease is accomplished by cultural practices and the use of fungicides (Eck, 1988). Evaluation of blueberry cultivars and breeding selections for resistance to fruit infection by *M. vaccinii-corymbosi* has been hampered by the lack of a consistent supply of conidia for making controlled inoculations. In southern New Jersey, conidia are produced on blighted shoots of infected plants only from late April through the third week of May. Since these naturally produced spores have a limited period of viability (Batra, 1983), the season for evaluating blueberry genotypes for resistance is short. If conidia could be reliably produced in vitro, controlled inoculations could be carried out at any time of year that blueberry plants could be induced to bloom.

Batra (1983) reported production of conidia of *M. vaccinii-corymbosi* in vitro on rabbit-food agar. In our initial attempts to produce conidia in vitro we were unable to induce conidia on rabbit-food agar using the temperature, light, and humidity conditions specified by Batra (1983). Other commercially available agar media also failed to stimulate conidia production. Filter paper disks had been shown to induce sporulation in other fungi (Dhingra and Sinclair, 1985). We modified this idea by placing cellulose acetate filter membranes on the surface of an agar medium prior to inoculation.

Batra (1983) determined that 15 °C and total darkness were optimal conditions for

conidia production by isolates of *M. vaccinii-corymbosi* grown on rabbit-food agar. However, a closely related species with an analogous life cycle, *M. oxycocci* (Woronin) Honey (the cause of cotton-ball disease in cranberry, *V. macrocarpon* Ait.), sporulated better under conditions of continuous illumination vs. alternating light and dark (Harada, 1977). Batra (1991), however, was unable to induce conidiation of *M. oxycocci* in culture. Our objectives were to evaluate the use of cellulose acetate membranes to enhance conidia production in *M. vaccinii-corymbosi*, and to determine optimal environmental conditions for sporulation.

Materials and Methods

The isolates of the *M. vaccinii-corymbosi* used in this study were acquired from P.V. Oudemans, Rutgers Univ. Single-conidial spore isolates were originally taken from cultures grown from surface-sterilized mummies placed onto potato dextrose agar (Becton Dickinson Microbiological Systems, Cockeysville, Md.). For assessment of the effects of cellulose membranes, two isolates, one from New Jersey and one from North Carolina, were grown on potato dextrose agar (PDA) at room temperature and ambient light. Plugs (5 × 5 mm) were taken from the outer edge of 1- to 2-week-old cultures with a cork borer and placed either directly on V-8 juice agar or on a 47-mm cellulose acetate membrane (Gelman Sciences, Ann Arbor, Mich.) placed on the surface of the V-8 juice agar (200 mL V-8, 800 mL H₂O, 3 g CaCO₃, 15 g agar). Dishes were then incubated for 1 week under one of three different environmental conditions: 1) room temperature (22 °C) with ambient room light (≈10 h fluorescent light/day); 2) room temperature (22 °C) and darkness; or 3) an incubator at 20 °C with 16 h fluorescent light (59 μE·s⁻¹·m⁻²).

For each environment evaluated, ten 100-mm-diameter petri dishes were initiated with each isolate, five with and five without a filter, and two sequential runs were conducted. To evaluate conidium production, 3 mL of distilled water containing 1% (v/v) Tween 20 was placed in each dish and the surface of the culture agitated with a rubber policeman. The spore suspension was then poured into a test tube containing two drops of lactophenol-cotton blue, then refrigerated until counted. Twelve subsamples were counted from each dish with a hemacytometer and their average computed.

Five isolates of *M. vaccinii-corymbosi* were selected to investigate the effect of temperature and light on conidium production on cellulose acetate membranes. Using similar starting conditions to those described above, the environmental treatments evaluated were as follows: 1) 15 °C + 24 h continuous fluorescent light (59 μE·s⁻¹·m⁻²), 2) 15 °C + 24 h darkness, 3) 22 °C (room temperature) + 10 h light (ambient daylight and dark cycles), and (d) 22 °C + 24 h darkness. Five dishes of each *M. vaccinii-corymbosi* isolate were randomly placed in each environment and incubated for 2 weeks. The experiment was run five times. Conidia per dish were counted as described previously. For all analyses, spore counts were transformed by converting to log₁₀(spore count + 1), and analyses of variance were run on the transformed data.

Results and Discussion

In vitro conidia production by *M. vaccinii-corymbosi* was significantly enhanced by cellulose acetate membranes placed on the surface of V-8 juice agar for both isolates tested, compared to V-8 juice agar alone (Fig. 1). A highly significant increase ($F = 83.28$, $P < 0.001$) in conidium production occurred when the cellulose acetate membrane was used. This enhancement represented an average 10-fold increase in conidia production. The increased production with the use of membranes occurred for the isolates at all three environments tested. The presence or absence of the membrane interacted significantly with environment ($F = 8.83$, $P = 0.003$) and isolate ($F = 9.77$, $P = 0.002$), but environment did not interact with isolates ($F = 1.55$, $P = 0.21$). Although this initial trial was primarily intended to evaluate the effect of membranes on conidia production and determine if the effect occurred under all environments, at the same time it allowed a preliminary evaluation of which environments optimize conidia production. Averaged across membrane and isolate, conidial production under illuminated environments (i.e., 20 °C + 16 h light and 22 °C + 10 h light, averaging 166,000 and 209,000 conidia/plate, respectively) were not significantly different, but were significantly different from production under the 22 °C + 24 h dark conditions (63,000 conidia/plate).

The second study of temperature and light indicated that both factors affected conidia production. A significant response to environments was shown by the group of five isolates

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($F = 14.46$, $P \leq 0.001$) (Fig. 2). In this evaluation, warmer temperatures promoted better conidia production in that two environments, 22 °C + 10 h light and 22 °C + 24 h light had the highest levels of production, but were not significantly different from 15 °C + 24 h dark. Similarly, illuminated environments were detrimental at any temperature, but were not significantly different from 15 °C + 24 h dark. In this second study there was significant interaction between isolate and environments, and no single environment maximized conidium production for all isolates (data not shown). The two studies are contradictory since the study with and without cellulose membranes found growth in illuminated environments superior to growth in the unilluminated environment. Hence, light environment seemed to produce no consistent results.

Variation in conidium production was evident among and within the seven isolates used in these first two studies. To further investigate this, 55 isolates from various sources were rated visually for sporulation on cellulose acetate membranes under 22 °C + 10 h light (the best conditions in the initial study). In Run 1 large differences were noted in conidium production (data not shown). When the test was repeated within one month of Run 1, many of the isolates showed decreased sporulation, with some isolates shifting from profuse sporulation to none. One isolate failed to sporulate in Run 1; 15 isolates failed in Run 2. Such variation was also noted by Batra (1983). Nonetheless, with the use of membranes, it was possible to assay quantity and reliability of conidium production, and three of 55 isolates (6%) were identified as consistently having very high conidia production. We have successfully used conidia produced in the described manner to infect blueberry flowers in a study of fruit infection resistance in diploid blueberry species (Stretch et al., 2000) and have achieved fruit infection rates as high as 47%.

More work is desirable if fully reliable conidia production for experimental purposes is to be achieved. Nonetheless, the current work is an incremental step forward in the understanding of the sporulation conditions needed for this fungus. The use of cellulose acetate membranes consistently improved conidia production under most conditions, and their use should allow the production of conidia on demand for resistance testing if sufficient

numbers of isolates can be evaluated initially to identify high producing isolates.

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Fig. 1. Effects of cellulose acetate membranes and three environments on conidium production by two isolates of *Monilinia vaccinii-corymbosi*. Values represent an average of two runs $\times$ two isolates $\times$ 10 plates $\times$ 12 samples/plate. Solid bars represent cultures with membranes; patterned bars represent cultures without membranes. Significant differences between membrane treatments were evaluated by *t* tests at $P = 0.05$; * indicates means within pair are significantly different.

Fig. 2. Effect of temperature and light on sporulation of five isolates of *M. vaccinii-corymbosi* growing on V-8 juice agar with cellulose acetate membranes. Values represent an average of five runs $\times$ five isolates $\times$ five plates $\times$ 12 samples/plate. Significant differences among means were determined by ANOVA contrasts. Bars with no common letters are significantly different at $P = 0.05$.