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Relationships Between Blueberry Flower Age, Pollination, and Conidial Infection by *Monilinia vaccinii-corymbosi*

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**ABSTRACT**


*Monilinia vaccinii-corymbosi* infects open blueberry flowers via the gynoecial pathway, leading to mummification of the developing fruit. To determine the effect of flower age on infection, stigmata were inoculated with conidia of *M. vaccinii-corymbosi* between 0 and 5 days after anthesis, fungal growth rates through the stylar canal were measured in detached flowers in the laboratory, and fruit disease incidence was determined in plants grown in the greenhouse. Hyphal growth rates were greatest in flowers inoculated on the day of anthesis, declined linearly with increasing flower age at inoculation (*r* = 0.921; *P* < 0.0001; *n* = 12), and were unaffected by the presence or absence of pollen applied at the time of inoculation. In greenhouse-grown plants, the percentage of infected fruit decreased exponentially with increasing flower age at inoculation (*R* = 0.878; *P* = 0.0057; *n* = 10), with disease incidence ranging from 76.4% for flowers inoculated on the day of anthesis to 15.5% for those inoculated 4 days later. Fruit disease incidence in the greenhouse was linearly correlated with hyphal growth rates in detached flowers (*r* = 0.985; *P* < 0.0001; *n* = 9), justifying the use of detached flowers when investigating gynoecial infection by *M. vaccinii-corymbosi*. In separate experiments, the effects of timing and sequence of pollination and inoculation on hyphal growth rates through the stylar canal and on disease incidence were investigated. Application of pollen to detached flowers 1 or 2 days before inoculation reduced hyphal growth rates by between 14.0 and 42.9% compared with flowers that received pollen and conidia simultaneously. Similarly, reductions in fruit disease incidence by between 9.5 and 18.3% were observed on greenhouse-grown plants for pollination-to-inoculation intervals ranging from 1 to 4 days. These results document that newly opened flowers are most susceptible to infection by *M. vaccinii-corymbosi* and that fruit disease incidence is reduced if pollination occurs at least 1 day before inoculation. Strategies that lead to early pollination of newly opened flowers may be useful for managing mummy berry disease in the field.

*Additional keywords: Vaccinium ashei, V. corymbosum.*

In spite of intensive control measures (21,22), mummy berry disease, caused by the discomycete *Monilinia vaccinii-corymbosi*, is a major problem in blueberry production in North America (8). The pathogen’s life cycle is strongly synchronized with the phenology of its host (12–14) and consists of two distinct monocycles; a primary infection phase initiated by ascospores in which young, vegetative tissues are blighted, and a secondary phase involving fruit infection by conidia via the gynoecial pathway (1,9,28). The latter phase begins when conidia deposited on the stigmatic surfaces of open flowers by insects or wind (2,28) germinate and the fungal hyphae ingress into the ovary via the stylar canal, traversing the same path as the pollen tubes (15,24,28). Previous reports suggest that hyphae reach the ovary within 7 days after inoculation, initially entering the locules before invading the fruit mesocarp (15,24). Mycelia stromatize at later stages of fruit development, resulting in fruit mummification (1,15,24). Mummified fruit fall to the ground where they serve as survival structures (4,28).

Although the gynoecial infection phase of *M. vaccinii-corymbosi* has been the subject of detailed histological studies (15,24), several questions regarding the biology of this process remain unanswered. At a very basic level, for example, there is no quantitative information on how conidial infection is affected by flower age. For pollen, which uses the same pathway as *M. vaccinii-corymbosi* to enter the ovary, flower receptivity (defined as the percentage of germinating pollen on the stigmatic surface or as fruit set resulting from pollination at different times after anthesis) changes considerably as flower age increases (16,29), with optimal receptivity to pollen occurring 1 to 2 days after anthesis in most blueberry cultivars (29). If flower receptivity to conidia is governed by a similar phenomenon, information relating risk of infection to flower age would be useful in determining the periods of greatest host susceptibility for better timing of disease management practices.

A second question is related to the potential for interactions between fungal hyphae and pollen tubes as they advance through the gynoecium. Theoretically, such interactions could be synergistic, in which case hyphae, pollen tubes, or both grow more rapidly as they traverse the stylar canal, or antagonistic, whereby the growth of either one or both is inhibited. Synergistic effects on fungal growth could occur, for example, if postpollination stigmatic exudation (10,11,18,23) provides a moisture or nutrient source for conidial germination and hyphal growth. Antagonistic effects on fungal growth, on the other hand, could arise from hyphae and pollen tubes competing for the same resources, e.g., finite space in the stylar canal. In field conditions, pollen and conidia may arrive on the stigmatic surface simultaneously or at different times, thereby adding another dimension to their interaction. For example, successful pollination preceding the arrival of conidia may trigger a style “shut-down” such as has been observed in pearl millet (*Pennisetum glaucum*) (25,26), thereby preventing hyphal ingress into the ovary.

Potential interactions among flower age, pollen, and conidia of *M. vaccinii-corymbosi* such as those outlined above could influence the likelihood of infection and the resulting disease incidence
in the mummy berry pathosystem. The objective of this study was, therefore, to determine the effects of flower age and of timing and sequence of pollination and inoculation on infection of blueberry flowers by *M. vaccinii-corymbosi*. An abstract summarizing preliminary results has been published (17).

**MATERIALS AND METHODS**

**Plant and pathogen culture.** Two- and three-year-old blueberry plants (*Vaccinium ashei* cvs. Tifblue and Powderblue; *V. corymbosum* cvs. Jersey and Bluecrop) in 12-liter pots were maintained in a cold room (7°C) or outdoors during winter for vernalization before being moved into a greenhouse with minimum and maximum temperatures of 18 ± 5°C and 25 ± 5°C, respectively, to induce flowering. The duration of exposure to vernalizing temperatures and the number of plants transferred to the greenhouse were varied to obtain a staggered bloom period, thereby ensuring that flowers were available for several months. In all experiments with *V. ashei*, pollen of cv. Powderblue was used to pollinate cv. Tifblue flowers; in experiments with *V. corymbosum*, pollen of cv. Bluecrop was used with flowers of cv. Jersey. Because the latter species has a high level of self-fertility, flowers of cv. Jersey were emasculated before pollination and inoculation. Two isolates of *M. vaccinii-corymbosi* obtained from the two different *Vaccinium* spp. were used, and growth conditions that best favor conidial production were adopted for each of the fungal isolates. isolate MBA-34 (originally isolated from infected fruit of *V. ashei*) was used for inoculation of cv. Tifblue, while MVC-4 (a single-ascoспорate isolate from apothecia formed on a pseudosclerotium from infected fruit of *V. corymbosum*) was used for inoculation of cv. Jersey. For conidial production, MBA-34 was grown for 2 weeks on half-strength oatmeal agar at 20°C with 8 h of light (29 to 45 μmol m⁻² s⁻¹). The medium was prepared by mixing 17.5 g of iron- and zinc-fortified single-grain oatmeal (Gerber, Fremont, MI) with 10 g of agar in 750 ml of deionized water (dH₂O) and autoclaving for 30 min. Conidia of MVC-4 were produced on cellulose membranes on V-8 juice agar as described previously (3).

**Effect of flower age on infection. Detached flowers.** Unopened individual flowers of cv. Tifblue were monitored daily to determine the date when flowers opened (referred to as day of anthesis hereafter), at which time they were detached and placed, the stigma facing upward, in 96-well microtiter plates containing 200 μl of dH₂O per well. Microrotter plates were maintained at room temperature (25°C) with 12 h light (34 to 55 μmol m⁻² s⁻¹) in a transparent plastic box, the bottom of which was lined with moistened tissue paper to maintain high humidity. Flowers were inoculated 0, 1, 2, 3, 4, or 5 days later with conidia of *M. vaccinii-corymbosi* collected with a hypodermic needle from the edge of a sporulating culture. Conidia readily adhered to the surface of the needle, allowing their transfer directly onto the stig mata without the need for a suspension medium, thereby mimicking transfer by insect vectors. Inoculation was carried out by positioning the microrotter plates under a dissecting microscope (×50) and applying conidia to the stigmata of individual flowers with the needle, taking care not to injure the stigmatic surface. This procedure resulted in approximately 50 conidia applied to each stigma. Immediately following inoculation, flowers were either pollinated (with approximately 50 pollen grains per stigma applied with a transfer needle) or not pollinated; application of pollen immediately following inoculation will be referred to as simultaneous inoculation and pollination henceforth. Treatments were applied in a split-plot design with flower age (days after anthesis) as the main-plot and pollination as the subplot, with eight flowers per treatment combination. The experiment was repeated seven times. Four days after inoculation, corollas were removed from the flowers and pistils were fixed overnight at room temperature in a solution containing 10% formalin, 8% glacial acetic acid, 50% ethanol, and 32% dH₂O per volume. Following fixation, pistils were rinsed twice in sterile dH₂O before clearing and further fixation for 2 h at 60°C in 0.3% trichloroacetic acid dissolved in a 3:1 vol/vol mixture of 95% ethanol and chloroform. Pistils were then rinsed at least twice in sterile dH₂O. For microscopic examination, pistils were stained in 0.1% decolorized aniline blue dissolved in 0.1 M K₃PO₄ (pH 12) and then rinsed twice in dH₂O. Styles were detached from the ovaries, split open longitudinally on a glass microscope slide, and observed for fungal growth with a Nikon epifluorescence microscope (Nikon, Tokyo) with a UV-2A dichoric mirror (excitation filter 330 to 380 nm, barrier filter 420 nm) and two neutral density filters.

Hyphal growth rates of *M. vaccinii-corymbosi* through the stylar canal were calculated from length measurements of the 10 longest hyphae in each style; fewer hyphae were measured if less than 10 hyphae penetrated into the stylar canal. For each treatment combination, means and standard errors (calculated from the eight experiments) of hyphal growth rate were plotted against flower age at inoculation, and the relationship between the two variables was analyzed by linear regression analysis (SAS version 6.12; SAS Institute, Cary, NC). Two separate regression models were fitted, and slopes and intercepts were compared (α = 0.05) to determine whether differences existed between treatments receiving inoculum only and those receiving inoculum and pollen simultaneously. In the absence of significant differences between these treatments, data were pooled and a single regression was fitted.

**Attached flowers.** Floral clusters of cv. Tifblue containing unopened flowers were selected, and for each individual flower within a cluster, the time of anthesis was noted and marked on the corolla with an indelible pen. Individual flowers were inoculated 0, 1, 2, 3, or 4 days after anthesis by gently rubbing their stigmata with mycelial plugs taken from a sporulating section of a culture of *M. vaccinii-corymbosi*. Immediately following inoculation, all flowers were pollinated with a transfer needle as described previously. There were 50 to 70 flowers per flower age for the first replicate and 70 to 104 flowers in the remaining seven replicates. The experiment was repeated once with cv. Jersey (three replicates with 50 to 70 flowers per treatment).

For each treatment, fruit set was determined as percentage of flowers pollinated. Individual fruit were harvested as they matured, dissected transversely, and examined with a dissecting microscope to determine infection based on the presence of mycelia or stromata of *M. vaccinii-corymbosi* (20). Means and standard errors of disease incidence (percentage of infected fruit) were calculated and plotted against flower age at inoculation. Nonlinear regression analysis was used to describe the relationship between the two variables (SigmaPlot version 7.101, SPSS Science, Chicago).

**Effects of timing and sequence of pollination and inoculation. Detached flowers.** Flowers were produced in the greenhouse and detached on the day of anthesis. Using the techniques described above, all flowers were pollinated on the second day after anthesis, and conidia of *M. vaccinii-corymbosi* were applied either 1 or 2 days before pollination (conidia-pollination treatment) or 1 or 2 days after pollination (pollen-conidia treatment). For comparison, a treatment in which conidia and pollen were applied simultaneously 2 days after anthesis was also included. Treatments were applied to experimental units consisting of 10 flowers, and the experiment was carried out eight times with cv. Tifblue and four times with cv. Jersey. Flowers were fixed, cleared, stained, and examined as described previously. Hyphal growth rates of *M. vaccinii-corymbosi* through the stylar canal were determined for each treatment and replication. Because inoculum was applied to flowers of different ages, effects of timing and sequence of pollination and inoculation on hyphal growth rates were confounded with flower age effects. For each treatment and replication, the latter effect was corrected for using the regression equation developed previously for relating hyphal growth rate to...
flower age at the time of inoculation (Fig. 1). Differences between these corrected growth rates and those observed in flowers receiving conidia and pollen simultaneously 2 days after anthesis were then calculated and expressed as relative changes (percent increase or decrease) due to the conidia-pollen and pollen-conidia treatments. In addition, $t$ tests were applied to determine whether these changes in growth rate were significantly different from zero ($\alpha = 0.05$).

**Attached flowers.** Experiments with attached flowers focused on the question of whether pollination preceding inoculation could reduce the incidence of fruit infection. Using the techniques described previously, individual flowers were pollinated on the day of anthesis and inoculated either 1 or 2 (short pollination-to-inoculation interval) or 3 or 4 days (long pollination-to-inoculation interval) later. A treatment in which conidia and pollen were applied simultaneously at anthesis also was included for comparison. At least 70 flowers were used for each treatment, and the experiment was replicated eight times with cv. Tifblue and repeated with three replicates of cv. Jersey. Fruit were harvested as they matured and dissected to determine disease incidence for each treatment and replication. Because inoculum was applied to flowers of different ages, effects of pollination-to-inoculation interval on disease incidence were confounded with flower age effects. For each treatment and replication, the latter effect was corrected for using the regression equation developed previously for relating disease incidence to flower age at the time of inoculation (Fig. 2). Differences between these corrected disease incidence values and those observed in flowers receiving conidia and pollen simultaneously at anthesis were then calculated and expressed as relative changes (percent increase or decrease) due to the short and long pollination-to-inoculation intervals. In addition, $t$ tests were applied to determine whether these changes in disease incidence were significantly different from zero.

**Relationship between measurements on attached and detached flowers.** This analysis utilized data from the experiments described above to determine whether hyphal growth rate in detached flowers could predict fruit disease incidence in attached flowers. Values of disease incidence obtained in the attached flower experiments were plotted against hyphal growth rates measured in detached flowers that had received equivalent inoculation and pollination treatments. Linear regression analysis was used to determine the strength and significance of the relationship between the two variables.

**RESULTS**

**Effect of flower age on infection.** **Attached flowers.** Conidia of *M. vaccinii-corymbosi* deposited on the stigmatic surface germinated within 24 to 36 h irrespective of the presence or absence of pollen. In >90% of conidia, germination was polar and produced single germ tubes from which hyphae were formed that

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**Fig. 1.** Effect of flower age at inoculation on hyphal growth rates in the stylar canals of detached blueberry flowers inoculated with conidia of *Monilinia vaccinii-corymbosi* with ($\Delta$) or without (・) simultaneous application of pollen. Values are means and standard errors of eight replicates, with eight styles examined per replicate and 10 hyphae measured per style. The dashed line represents a linear regression relating growth rate ($g$) to flower age ($t$) fitted to both data sets ($g = 982.1 - 180.1 t; r = 0.921; P < 0.0001$).

**Fig. 2.** Effect of flower age at inoculation on incidence of fruit infection following inoculation of blueberry flowers with conidia of *Monilinia vaccinii-corymbosi* and simultaneous application of pollen. Values are means and standard errors of eight and three replicates for cvs. Tifblue (○) and Jersey (□), respectively, with >50 flowers inoculated per replicate. *M. vaccinii-corymbosi* isolate MBA-34 was used to inoculate cv. Tifblue flowers, and isolate MVC-4 was used with flowers of cv. Jersey. The dashed line represents a nonlinear regression relating disease incidence ($y$) to flower age ($t$) fitted to both data sets ($y = 23.79 + 42.30e^{-0.074t}; R = 0.878; P < 0.0057$).

**Fig. 3.** Relative reduction in hyphal growth rates of *Monilinia vaccinii-corymbosi* in the stylar canals of detached blueberry flowers pollinated either 1 or 2 days before inoculation (pollination-inoculation treatment) or 1 or 2 days after inoculation (inoculation-pollination treatment), relative to flowers receiving pollen and conidia simultaneously. All flowers were pollinated 2 days after anthesis. Because flowers were inoculated at different times after anthesis, hyphal growth was corrected for flower age using the regression depicted in Figure 1. Values are means and standard errors of eight (cv. Tifblue) or four (cv. Jersey) replicates, with eight styles examined per replicate and 10 hyphae measured per style. Numbers above each bar are $P$ values for a $t$ test for the null hypothesis that mean growth reduction is zero. Actual growth rates in flowers receiving pollen and conidia simultaneously were 577.0 and 485.0 $\mu$m day$^{-1}$ for cvs. Tifblue and Jersey, respectively.
Inoculated flowers. Application of pollen to attached flowers before inoculation reduced infection by _M. vaccinii-corymbosi_. Indeed, significant (\(P < 0.0001\) for both cultivars) reductions in the incidence of infected fruit by between 9.5 and 18.3%, compared with the treatment receiving pollen and inoculum simultaneously, were observed when flowers were inoculated between 1 and 4 days before inoculation (Fig. 4). The decrease in disease incidence associated with pollination before inoculation was similar for the two pollination-to-inoculation intervals tested (\(P = 0.142\)).

**Relationship between measurements on attached and detached flowers.** Fruit disease incidence \(y\) in greenhouse-grown plants was positively correlated \((y = 0.065g; r = 0.985; P < 0.0001; n = 9)\) with hyphal growth rates \(g\) in detached flowers receiving equivalent treatments (Fig. 5). The regression equation was constrained to pass through the origin after an initial \(t\) test revealed that the intercept was not significantly different from zero \((P = 0.630)\).

**DISCUSSION**

Earlier studies on infection of blueberry flowers by _M. vaccinii-corymbosi_ provided detailed information on histological aspects of the gynoecial infection process (15,24,28). However, relationships between flower age, pollen, and conidia during this process have not been investigated previously. In the present study, we documented that both flower age and the timing and sequence of inoculation and pollination affect infection by _M. vaccinii-corymbosi_. Infection was reduced significantly by increasing flower age at inoculation and by application of pollen at least 1 day before inoculation. We further showed that hyphal growth rate in detached flowers is a good predictor of disease incidence in attached flowers; this justifies the use of detached flowers as a surrogate and facilitates further studies on gynoecial infection of _M. vaccinii-corymbosi_ in controlled laboratory experiments.

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**Fig. 4.** Relative reduction in the incidence of fruit infection following inoculation of blueberry flowers with conidia of _Monilinia vaccinii-corymbosi_ when pollination was applied at different periods before inoculation, relative to flowers receiving pollen and conidia simultaneously. All flowers were pollinated on the day of anthesis, and the period between pollination and inoculation was manipulated by inoculating either 1 or 2 days or 3 or 4 days later. Because flowers were inoculated at different times after anthesis, disease incidence was corrected for flower age using the regression depicted in Figure 2. Values are means and standard errors of eight and three replicates for cvs. Tifblue and Jersey, respectively, with >50 flowers inoculated per replicate. Actual disease incidence in flowers receiving pollen and conidia simultaneously was 56.9 and 76.4% for cvs. Tifblue and Jersey, respectively.

**Fig. 5.** Relationship between the incidence of infected fruit in greenhouse-grown blueberry plants and hyphal growth rates of _Monilinia vaccinii-corymbosi_ in the stylar canals of detached flowers receiving equivalent inoculation and pollination treatments. For hyphal growth rates \(g\), values are means and standard errors of eight (cv. Tifblue, •) or four (cv. Jersey, ○) replicates, with eight styles examined per replicate and 10 hyphae measured per style. For disease incidence \(y\), values are means and standard errors of eight and three replicates for cvs. Tifblue and Jersey, respectively, with >50 flowers inoculated per replicate. The corresponding regression equation is \(y = 0.065g (r = 0.985; P < 0.0001)\).
Evidence for reduced hyphal growth when pollination preceded inoculation, together with the reduced disease incidence observed when inoculation was delayed relative to pollination, suggests that early and improved pollination of newly opened flowers may be a useful strategy for managing mummy berry disease in the field. This could be accomplished, for example, by use of supplemental bees or by placing more emphasis on pollen compatibility and on synchronicity in bloom periods when selecting cultivars for planting of self-sterile blueberry species such as V. ashei. Use of supplemental bees to enhance pollination poses an intriguing dilemma, however, in that bees also serve as important vectors for conidia of M. vaccinii-corymbosi (2).

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**LITERATURE CITED**


