Effect of Triforine on Pollen Germination and Fruit Set in Highbush Blueberry

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ABSTRACT

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Pollen of 13 highbush blueberry (*Vaccinium corymbosum* L.) cultivars germinated on 9% sucrose agar but was inhibited when the medium contained 10–50 μg (active ingredient) of the fungicide triforine per milliliter. Triforine applied directly to the stigma at 3,488 μg/ml (the concentration used in suspensions applied aerially to control the mummy berry disease) prevented pollen germination. Inhibition was greatest when triforine was applied to stigmas 2 hr before pollination and decreased as the interval between fungicide application and pollination lengthened. When sprayed on flower parts inside the corolla tube, triforine reduced germination of undehisced pollen; concentrations above 500 μg/ml caused stickiness and necrosis of the corolla tube and impaired the release of pollen. Blueberry bushes in the field sprayed during bloom to simulate aerial application of triforine did not develop blossom necrosis; pollen from sprayed flowers germinated readily on sucrose agar; and fruit set and berry development were not affected. Although it is toxic to blueberry pollen, triforine has no adverse effect when applied during bloom to control mummy berry.

Additional key words: Funginex, *Monilinia vaccinii-corymbosi*

Mummy berry, caused by *Monilinia vaccinii-corymbosi* (Reade) Honey, is a serious disease of cultivated highbush blueberry (*Vaccinium corymbosum* L.) throughout North America. For effective control, fungicides must be applied from budbreak through full bloom (4). The mummy berry stage results when conidia germinate on the stigma of the flower and the pathogen grows through the style into the ovary, eventually colonizing developing berry tissues to form a sclerotium (16).

Many researchers have reported the adverse effects of fungicides and other pesticides on pollen germination; a brief review of this literature is contained in Church and Williams (6). Fungicides applied during bloom for disease control reduced fruit set in cranberry (20) and apple (9,15) and increased the incidence of malformed strawberry fruit because of the reduction in achene set (1,12).

Triforine at recommended rates drastically lowered germination of apple pollen (6,7). Unlike apples, where a 20% fruit set can produce large yields, blueberries require a fruit set of more than 80% for a good commercial crop (21). Since 1977, triforine (Funginex) has been used in Washington to control mummy berry under emergency use registrations granted each year by the U.S. Environmental Protection Agency.

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Given the need for high fruit set and the possibility that this new fungicide is toxic to blueberry pollen, this research was initiated to determine triforine's toxicity to blueberry pollen and its effect on fruit set when applied during bloom for disease control. A preliminary report has been published (3).

MATERIALS AND METHODS

In vitro tests. Water agar and 18% sucrose agar (w/v), both containing 5% agar (w/v), were prepared with glass-distilled water, autoclaved, cooled to 45 C, mixed with equal volumes of aqueous suspensions of triforine, and dispensed into petri plates 9 cm in diameter at the rate of 18 ml per plate. Plates were prepared with final triforine concentrations of 0, 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1,000, and 5,000 μg of active ingredient (a.i.) per milliliter and were used immediately. A commercial emulsifiable concentrate formulation of triforine (CME 74770; 186 g a.i./L) was used in all experiments.

Pollen was obtained from blueberry flowers collected from a field planting, or for one experiment from bushes potted in the greenhouse. To seed plates with pollen, a blossom was held by its pedicel with forceps in an inverted position over the agar surface and the forceps were tapped with a pencil. Each plate was seeded with pollen from two blossoms. Except where noted, seeded plates were incubated at 27 C in darkness for 18 hr. After incubation, the pollen was killed and stained with phenolic rose bengal (1% rose bengal, 5% phenol, and 0.01% calcium chloride [all w/v]).

Blueberry pollen is shed as tetrads of four pollen grains, each grain capable of producing a pollen tube. The percentage of tetrads with at least one pollen tube and the percentage of pollen grains germinating were determined for at least 50 pollen tetrads per plate. The lengths of 25 pollen tubes were measured per plate. At least two replicate plates per treatment were included in all in vitro experiments.

In vivo tests. Unpollinated receptive stigmas were obtained by selecting fully developed blossoms with corolla tubes just about to open. The corolla tube and stamens were carefully removed with forceps, and the pedicel was placed in moist sand in a deep petri dish to hold the flower upright. A 0.25 μL droplet of water or an aqueous suspension of triforine was applied to each stigma with a 50 μL syringe mounted in a syringe microburette (Micro-Metric Instrument Co., Cleveland, OH 44125). The droplet was allowed to dry for 1 hr before pollination. In one experiment, pollen was applied to stigmas at various times before and after the application of water and triforine.

Flowers were pollinated by lightly touching the stigma to freshly collected pollen. Pollen from the cultivar Jersey was used to pollinate Concord flowers, and vice versa. Flowers were incubated at 20 C in darkness for 48 hr after pollination; 8–10 replicate blossoms were pollinated per treatment.

To assess pollen germination on the stigma and pollen tube growth in the style, the pistil was cut at the point of style attachment to the ovary and these tissues were prepared for fluorescence microscopy. The stigma and style were fixed, softened, and stained following Johnson (13), then gently squashed in glycerin and immediately examined with a Zeiss epifluorescent microscope with an ultraviolet light source (350–400 μm).

Because it was impossible to see the origin of all pollen tubes, only tetrad germination was determined in in vivo tests. Maximum pollen tube growth in the style was recorded as a percentage of the distance from the stigma to the ovary.

In the greenhouse, open blossoms on potted Concord bushes were sprayed with water or triforine suspensions. To spray the outside, a glass chromatography sprayer (Arthur H. Thomas Co., Philadelphia, PA 19106) was positioned so that the spray would not enter the

corolla, and flowers were sprayed to runoff. To spray the stamens and pistil, the sprayer was positioned at the opening of the corolla tube. Approximately 0.1 ml of the suspension was sprayed into each flower. Pollen was collected and assayed for germination on 9% sucrose agar (w/v) 24, 48, and 72 hr after spraying.

In field experiments, bushes were sprayed with triforine at the commercial aerial application rate of 1,754 ml/ha in 93.5 L of water with a knapsack-type sprayer. The concentration of triforine in this suspension was 3,488 μg/ml. Some branches were enclosed in plastic bags during spraying to serve as unsprayed controls.

In one experiment, pollen from flowers newly opened at the time of spraying was assayed for germination as described above at various times after spraying. In a second field experiment, newly opened blossoms of Jersey and Concord bushes were similarly sprayed to assess the effect of triforine on pollen deposition and germination as well as on fruit set and fruit development. Styles with attached stigmas were examined 12 days after spraying by fluorescence microscopy as outlined above. Fruit set was determined 1 mo after spraying, and fruit was hand-harvested in mid-July, when approximately 60% of the fruit was ripe.

No other chemicals were applied to field plots during the test period. A sheltered hygrothermograph was maintained at the field experiment site, and other pertinent weather data were collected at a weather station 4.8 km away.

RESULTS

In vitro tests. Triforine at 50 μg/ml prevented germination of pollen from the cultivar Bluecrop in both water agar and 9% sucrose agar (Table 1). Pollen remained ungerminated at this and higher triforine concentrations when incubation was lengthened from 18 to 36 and 72 hr. In the longer incubation periods, profuse pollen tube growth on control plates and plates with triforine concentrations of 5 μg/ml or less prevented collection of accurate data for these plates. Longer incubation of pollen on 9% sucrose agar containing 10 μg of triforine per milliliter significantly ($P = 0.05$) increased pollen tube length over that at 18 hr but had no effect on germination. Triforine concentrations of 0.1 and 0.5 μg/ml stimulated pollen cell germination and pollen tube growth, respectively, but only on the medium containing sucrose.

Triforine sensitivity of pollen from 13 commercial cultivars was determined with the in vitro method on 9% sucrose agar. Pollen from all 13 cultivars germinated readily on this medium. Triforine completely inhibited pollen germination of the cultivars Atlantic, Bluecrop, Bluetta, Collins, Earliblue, Pemberton, Rubel, Washington, and Weymouth at 50 μg/ml; of Concord, Ivanhoe, and Jersey at 100 μg/ml; and of Berkeley at 500 μg/ml. Fifty μg/ml reduced pollen grain germination in all cultivars by at least 95%. Triforine concentrations of 0.1 and 0.5 μg/ml stimulated pollen grain germination of Bluecrop, Bluetta, Berkeley, Earliblue, and Ivanhoe pollen but not that of the other cultivars.

In vivo tests. A triforine concentration of 10,000 μg/ml completely inhibited germination of Concord and Jersey pollen when applied directly to the stigma 1 hr before pollination. Germination of pollen from both cultivars was unaffected by concentrations of 1,000 μg/ml or less, and at least 10–15 pollen tubes were observed at the base of styles 48 hr after pollination. Triforine at 10,000 μg/ml did not cause necrosis of the stigma or any other phytotoxic effects. In subsequent experiments of this kind, triforine at 3,488 μg/ml prevented germination.

Triforine was toxic to pollen when applied to stigmas up to 24 hr before and after pollination. A concentration of 3,488 μg/ml completely inhibited germination of Concord pollen when applied 2 hr before pollination, and only 3.3% of the pollen tetrads germinated

Table 1. Germination of Bluecrop highbush blueberry pollen on triforine-amended media

Incubation time[y] (hr)	Triforine concentration (μg a.i./ml)	Water agar[x] Pollen germination (%)[z] Tetrads	Water agar[x] Pollen germination (%)[z] Grains	Water agar[x] Pollen tube length[z] (μm)	9% Sucrose agar Pollen germination (%)[z] Tetrads	9% Sucrose agar Pollen germination (%)[z] Grains	9% Sucrose agar Pollen tube length[z] (μm)
18	0.0	96 a	51 a	239 a	97 a	65 b	458 cd
	0.1	87 a	46 a	269 a	99 a	82 a	550 bc
	0.5	87 a	40 a	291 a	100 a	71 ab	707 ab
	1	88 a	31 a	282 a	100 a	74 ab	786 a
	5	91 a	38 a	227 a	97 a	63 b	336 de
	10	31 b	8 b	199 a	13 b	3 c	219 e
	50	0 c	0 b	0 b	0 c	0 c	0 f
36	10	...	...	...	49	15	308
	50	...	...	...	0	0	0
72	10	...	...	...	27	8	264
	50	...	...	...	0	0	0

[x] ... = no data (tests on water agar were not conducted at times other than 18 hr).
[y] Pollen-seeded agar incubated in darkness at 27 C.
[z] Values within columns followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

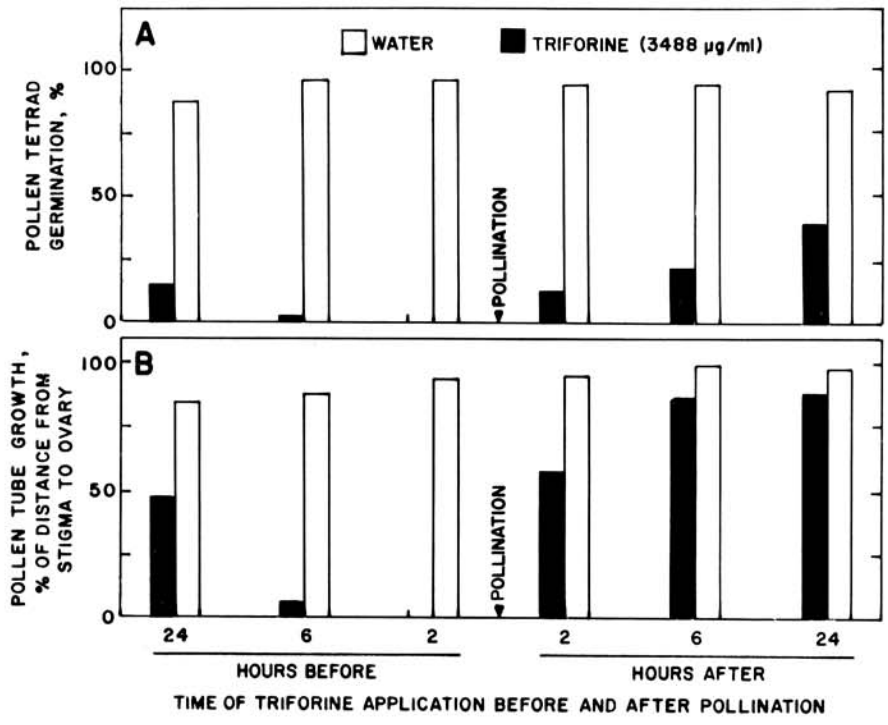

Fig. 1. Tetrad germination **(A)** and maximum pollen tube growth in the style **(B)** of Concord blueberry pollen when water or triforine at 3,488 μg/ml was applied to the stigma of Jersey blueberry flowers at various times before and after pollination. Data were recorded after pollen was incubated on stigmas for 48 hr at 20 C.

when the stigmas were treated 6 hr before pollination (Fig. 1A). Germination was reduced by at least 65% when stigmas were treated 2 hr before or 2, 6, or 24 hr after pollination. Results for pollen tube growth in the style paralleled those for germination, with the least growth occurring when the interval between triforine application and pollination was the shortest (Fig. 1B).

Triforine at concentrations up to 5,000 μg/ml applied to the outside surfaces of flowers on potted Concord blueberry bushes in the greenhouse had no adverse effect on pollen germination when pollen was assayed 24 and 48 hr after spraying. After 72 hr, however, pollen tetrad and grain germination were reduced 80 and 50%, respectively, when the outsides of flowers were sprayed with suspensions containing 100 μg/ml or more.

Directing suspensions containing 100 μg/ml or more into the corolla tube reduced pollen grain germination by at least 33% after 24 hr and lowered pollen tetrad germination by 75% after 72 hr. Applying triforine to the inside of the corolla tube, even at 5,000 μg/ml, did not completely inhibit pollen germination. Treating blossoms with suspensions of 500 μg/ml or more caused browning of the corolla, and treated flower parts became sticky. Triforine applied at these concentrations to stamens inside the flower impaired the release of pollen.

When newly opened Concord flowers in a commercial field were sprayed with triforine to simulate aerial application, neither pollen tetrad nor pollen grain germination was reduced after 0, 24, 48, and 72 hr. No necrosis of the corolla nor stickiness of flower parts was noted.

In a second test, abundant germinated pollen was observed on stigmas, with numerous pollen tubes penetrating to the ovaries of both Jersey and Concord blossoms 12 days after spraying, regardless of whether blossoms had been sprayed with triforine or not (Fig. 2). During this 12-day period, weather conditions were favorable for pollinator activity for 51 hr, of which 36 hr occurred within 4 days of spraying. Temperatures above 15 C with no rain and little wind were considered favorable for pollinator activity (19).

Fruit set exceeded 93% for both cultivars for sprayed as well as unsprayed flowers. Furthermore, triforine had no effect on the weight or diameter of ripe or green fruits, and the proportion of ripe to green berries was the same for sprayed and unsprayed blossoms.

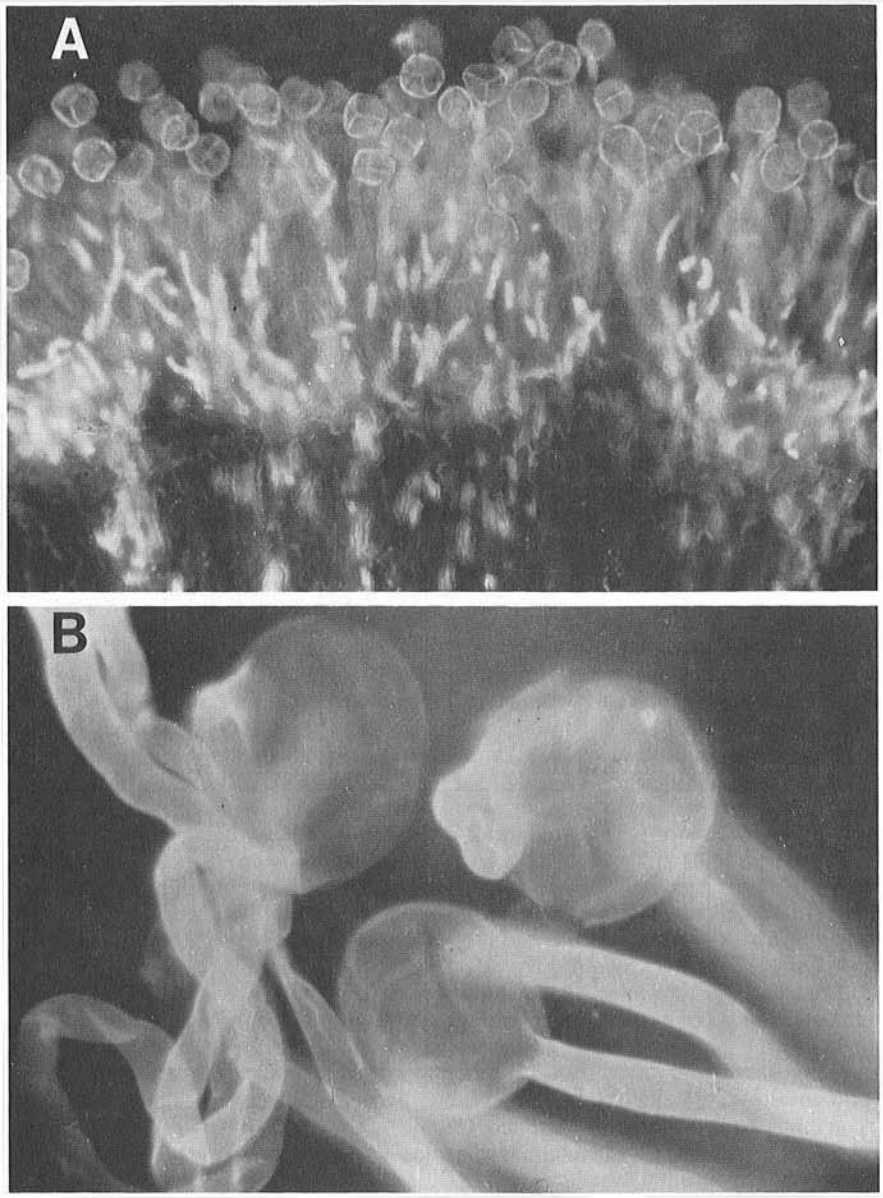

Fig. 2. Ultraviolet photomicrographs of **(A)** the stigma of an open-pollinated Jersey blueberry flower 12 days after newly opened unpollinated flowers were sprayed with triforine at 3,488 μg/ml and **(B)** close-up of tetrads on the stigma, showing pollen grain germination.

DISCUSSION

Pollen of highbush blueberry is very sensitive to the fungicide triforine—pollen from all 13 cultivars tested failed to germinate at concentrations markedly below that in the suspension used in aerial applications to control the mummy berry disease. Some low concentrations of triforine increased pollen grain germination and pollen tube growth, but only when sucrose was present in the medium. In other tests (P. R. Bristow, *unpublished*), adding elements (Ca, B, etc.) reported to be essential for germination of pollen from other plants (2,8) did not improve the germination of Bluecrop blueberry pollen over that in unamended sucrose agar. It is unlikely that minerals that may be in the CME 74770 formulation of triforine cause the stimulatory effect.

Considerably higher concentrations of triforine were required to prevent pollen germination in vivo than in vitro. The fact that plant tissues readily absorb this fungicide (10) may partially explain this difference. Absorption of triforine may also account for the reduced viability of undehisced pollen when the outside surfaces of flowers were sprayed in a greenhouse test.

Toxicity of several fungicides to apple pollen has been reported to be greatest when emasculated flowers are sprayed 2 hr after pollination (6). Results of two experiments suggest that the adverse effect on blueberry pollen of triforine on the stigma is of limited duration. When tested in vivo, triforine completely inhibited pollen germination only when applied to stigmas shortly before pollination. Bees were foraging when and after plants were sprayed in the field, and conditions for their activity were favorable for several days after spraying. Thus, pollen deposition probably occurred when triforine was most toxic. Nevertheless, fruit set and development were unaffected. If reduced pollen germination had decreased the number of ovules being fertilized, this should have been reflected in berry size (weight and diameter), because berry size is highly

correlated with seed number (11,18).

In field experiments, corollas did not turn brown, flowers did not become sticky, pollen viability was not reduced, and pollen release was not impaired, contrary to greenhouse tests when blossoms were sprayed with triforine from an atomizer. Impaired release of pollen most likely resulted from the plugging of the exit pore of some anther tubes. Differences in the results from greenhouse and field experiments and the volume of triforine suspensions applied to each flower in the greenhouse experiment suggest that little triforine is deposited on flowers in the field.

The pendant position of the blueberry flower and the corolla tube, which surrounds the stigma and anthers, apparently restricts the amount of triforine that reaches these flower parts. The protection provided by the corolla tube is illustrated by the fact that in artificial medium, ferbam reduced germination of both lowbush blueberry (14) and cranberry (20) pollen, but when the fungicide was sprayed on these crops during bloom, cranberry (exposed stigma) yield was reduced by 32%, while lowbush blueberry (corolla-protected) was unaffected. Photodecomposition of triforine on the the stigma may further reduce its concentration (5). The very little triforine that must reach the stigma of highbush blueberry flowers is, however, enough to control the mummy berry stage of the disease (4).

No more than two sprays of triforine would be made during bloom at the recommended interval between applications of 10–14 days. This, coupled with the knowledge that blueberry flowers remain receptive to pollen for up to 8 days (17) and the results of this study, provides more evidence that fruit set is not reduced when triforine is applied during bloom for disease control, even though it is toxic to blueberry pollen.

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