Reduction of *Botrytis cinerea* Infection on Petunia Flowers following Calcium Spray Applications

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Abstract. Botrytis blight on petunia flowers causes significant losses in the postharvest environment. Infection occurs during greenhouse production, and symptoms are expressed during transport. This phenomenon is termed petunia flower meltdown because of the rapid collapse of flower petal tissue as the plants are transported from the production greenhouse to the retail store. The objective of this study was to determine the effect of calcium (Ca) spray applications on botrytis blight severity in petunia flowers. For the first experiment, petunia ‘Pretty Grand Red’ plants were sprayed twice per week for 2 weeks with calcium chloride (CaCl₂) at rates of 0, 400, 800, and 1200 mg·L⁻¹ Ca. A fungicide (cyprodinil, 37.5%; fludioxonil, 25%) was used as an additional control treatment. Twenty-four hours after the last treatment, freshly opened flowers were harvested, placed into a humidity chamber with 99% relative humidity, and inoculated with a *Botrytis cinerea* spore suspension (1 × 10⁴ conidia/mL). Disease progression was recorded every 12 hours for 72 hours. The results showed a 96% reduction in botrytis blight severity as Ca concentration increased from 0 to 1200 mg·L⁻¹ Ca. The Ca treatments provided better disease control than the fungicide treatment because of the fungicide resistance of the isolate used in the study. A second experiment was performed to determine whether the beneficial response to CaCl₂ application was influenced by chlorine (Cl) or the electrical conductivity (EC) of the spray solutions, and no significant responses were observed. These studies prove Ca is the sole source of the reduction in botrytis blight severity following treatment with CaCl₂ sprays, and demonstrate the benefit of using Ca as a tool for the management of botrytis blight on petunia flowers.

Botrytis cinerea, the causal agent of botrytis blight, is a ubiquitous plant pathogen that infects more than 200 crop species worldwide. Although there are fungicides available

for botrytis blight management, many chemical classes have low efficacy as a result of the high levels of fungicide resistance in commercial greenhouses (Williamson et al., 2007). *Botrytis cinerea* becomes a significant threat in greenhouses during periods with relative humidity greater than 93% (Williamson et al., 2007) and temperatures between 10 and 20 °C; however, infection can also occur at temperatures ranging from 2 °C to above 25 °C (Elad and Shtienberg, 1995). Moderate temperatures and/or rainy weather during the spring bedding plant season affect retail sales negatively, which causes growers to hold flowering plants in the greenhouses. During this time, plants grow closer together, forming a denser canopy, and the oldest flowers begin to senesce and die. The senesced flowers then become more susceptible to *Botrytis* infection as the pathogen favors dying tissue. These conditions increase the amount of inoculum present when the plants are moved into the postharvest environment, resulting in the proliferation of botrytis blight infection

on petunia flowers and causing significant losses.

Botrytis cinerea produces cell wall-degrading enzymes that play an important role in tissue maceration. Polygalacturonases are the first cell wall-degrading enzymes produced by *B. cinerea* that bind with polygalacturonic acid (pectin material) in the middle lamella in the intercellular spaces of the plant (Cabanne and Donèche, 2002). Calcium may act as a competitive inhibitor for the binding site of pectin material, causing gel formation in the middle lamella as a result of Ca cross-linking in the pectin chains (Conway and Sams, 1984), resulting in polygalacturonic acid becoming less accessible spatially to polygalacturonase enzymes (Cabanne and Donèche, 2002).

Calcium deficiencies are common in horticultural crops (White and Broadley, 2003), and often result from inefficient uptake and distribution of Ca throughout different tissues (Poovaiah, 1988). Calcium uptake is passive via the mass flow of water through the plant and is dependent on transpiration. Flower buds and fruits are low-transpiring organs as a result of their low stomatal density and/or large volume-to-surface area ratio. Similarly, young leaves that are enclosed in heads, such as in head lettuce, have low transpiration rates, making them particularly susceptible to Ca deficiencies. Reproductive tissues (e.g., fruit and flowers) are mainly supplied with nutrients through the phloem, where transport of Ca is low (Marschner, 2012). Increasing the Ca concentration in the nutrient solution can provide sufficient Ca in leaves that transpire at relatively high rates throughout their life cycle, but not necessarily in low-transpiring organs (Marschner, 2012).

Numerous studies have examined the effect of Ca on botrytis blight on horticultural crops. In cut roses, increasing Ca concentration in the nutrient solutions increased Ca content of the petals and decreased botrytis blight severity (Baas et al., 2000; Bar-Tal et al., 2001; Volpin and Elad, 1991). Similarly, in potted roses, increasing Ca in the nutrient solution resulted in greater Ca in the flower petal tissue and reduced botrytis blight incidence (Starkey and Pedersen, 1997). Two similar studies evaluated preharvest spray applications of calcium sulfate on cut roses that reduced botrytis blight severity in flower petals (De Capdeville et al., 2005; Nabigol, 2012). Bract edge burn disorder of poinsettia begins as necrotic spots along the margin of the bracts, which is caused by a localized Ca deficiency (Strømme et al., 1994). The necrotic tissue can then be infected with *B. cinerea*, resulting in coalesced lesions that are termed bract edge burn (Barrett et al., 1995). This phenomenon can be reduced with weekly Ca spray applications during bract development (Woltz and Harbaugh, 1985). Calcium spray applications also increase the strength of the leaves of unrooted cuttings of poinsettia and geranium, and a subsequent reduction in botrytis blight severity was also observed in poinsettia leaves (Samarakoon et al., 2017a). In grapes, preharvest Ca spray

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applications significantly reduced storage rot from *B. cinerea* (Nigro et al., 2006). Post-harvest dips or vacuum infiltration of Ca on apples increased Ca content of apples and reduced decay caused by *B. cinerea* (Conway et al., 1993).

Calcium chloride spray solutions provide a means of increasing Ca content in plant tissues. However, the CaCl_2 solutions applied to plants can increase the EC/soluble salts, which have also been shown to suppress various fungal pathogens directly (Deliopoulos et al., 2010), leading to the importance of differentiating between Ca and EC effects. In addition, Cl is also supplied as chloride during CaCl_2 applications and may have an effect on *Botrytis* infection. For example, $300 \text{ mg}\cdot\text{L}^{-1}$ CaCl_2 and sodium chloride (NaCl) solutions similarly reduced the germination of *B. cinerea* spores (Boumaaza et al., 2015). Studies on *B. cinerea*, as well as other fungi, have been performed to evaluate the pathogen response to Cl foliar sprays with varying results. A study on grapes showed that spray applications of potassium chloride (KCl) and NaCl did not provide any reduction in the development of botrytis blight, whereas the CaCl_2 treatment provided significant reduction in botrytis blight development (Nigro et al., 2006). Kettlewell et al. (2000) investigated the use of KCl sprays for powdery mildew disease (*Erysiphe graminis*) of wheat and suggested an osmotic effect on the causal agent for disease reduction.

Based on the literature demonstrating the effect of Ca spray applications on leaf tissue strength and botrytis blight, we hypothesized that Ca may be beneficial for the management of petunia flower meltdown. The objectives of this study were to determine the effect of CaCl_2 spray applications on botrytis blight severity on petunia flowers and to determine whether any beneficial response observed was the result of Ca, Cl, and/or the EC of the spray solution.

Materials and Methods

Two experiments were conducted to quantify the effect of Ca spray applications on the resistance of petunia flowers to botrytis blight. The first experiment examined the response of harvested petunia flowers following spray applications of CaCl_2 . The second experiment was conducted to determine whether the responses to CaCl_2 treatments observed in Expt. 1 were the result of Ca, Cl, and/or the EC of the CaCl_2 solutions.

Botrytis handling procedures. To obtain a pure culture of a *Botrytis* isolate, petunia plants were received from a commercial grower, and plant tissue symptomatic of a *B. cinerea* infection was removed, placed in a plastic bag containing a moist paper towel, and incubated on a laboratory bench at 22°C until sporulation was observed within 1 to 5 d. A pure culture was obtained by isolating spores from the incubated plant tissue and plating them on a petri dish ($100 \times 15 \text{ mm}$) with potato dextrose agar (PDA) medium (Difco Laboratories, Sparks, MD) under sterile conditions. Mycelia from the first milli-

meter of the leading edge of the colony was transferred to new PDA plates. This process was repeated until a pure mycelium culture of a single *B. cinerea* isolate was obtained. Species identification was determined based on spore shape and color, and polymerase chain reaction analysis (Fernández-Ortuño et al., 2011).

For long-term storage, conidia were harvested by pipetting 3 mL sterile aqueous solution of 0.01% Tween 80 (Sigma-Aldrich Corporation, St. Louis, MO) and 15% glycerol onto the petri dish. The spore solution was then transferred to 2-mL cryogenic vials (Nalgene Corporation, Rochester, NY) and stored in an ultralow-temperature freezer at -80°C .

To obtain fresh spores for inoculation, the stored spores in cryogenic vials were retrieved from the -80°C freezer and the solution was pipetted onto PDA and allowed to grow for 7 to 10 d. From each incubated PDA plate, mature spores were placed into solution by pipetting 5 mL sterile deionized water onto the PDA plates and using a sterile stir rod to press lightly on spores to get them to release from the conidiophores and go into solution. The isolate was incubated until sporulation occurred and spores matured from clear to gray over 7 to 10 d. The spore solution was then pipetted from the PDA plate into sterile deionized water to prepare a suspension measured to 1×10^4 conidia/mL using a hemocytometer (Bright-line 3110; Hausser Scientific, Horsham, PA) by placing 25 μL on each side of the hemocytometer.

General procedures. Petunia plugs were received from a commercial grower (Expt. 1, *Petunia $\times$ hybrida* 'Pretty Grand Red'; Expt. 2, *Petunia $\times$ hybrida* 'Dreams Red') and transplanted in 1.4-L round containers containing a peat-based growing medium (Fafard 3B; Conrad Fafard, Inc., Agawam, MA) to provide a supply of flowers for the experiments. The plants were grown in a glass greenhouse at Clemson University, SC (lat. 34.7°N , long. 82.8°W), with the environment controlled by a climate-control computer (Argus Control Environmental Systems; White Rock, British Columbia, Canada). A constant liquid fertigation program was used, with Peter's Excel Cal-Mag Special (15N–2.2P–12.5K–5Ca–2Mg; Scotts-Sierra, Marysville, OH) providing $150 \text{ mg}\cdot\text{L}^{-1}$ N and $50 \text{ mg}\cdot\text{L}^{-1}$ Ca at each irrigation event.

Each experiment had two replications. For Expt. 1, the first replication occurred during October; the second replication occurred during November. The plants were grown with daylength extension lighting with metal halide lamps when solar radiation measured outdoors was less than $200 \text{ W}\cdot\text{m}^{-2}$ from 900 to 2400 HR to promote flowering of this facultative long-day plant during October and November. The daily light integrals in October and November were 16.0 ± 6.2 and $11.4 \pm 4.5 \text{ mol}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$, respectively, whereas the average daily temperatures were 22.1 ± 1.0 and $21.5 \pm 0.9^\circ\text{C}$ in October and November, respectively. For Expt. 2, the first replication took place in June and the second

in July. The experiment was conducted under the ambient photoperiod with no supplemental lighting. Plants were shaded with retractable curtains providing 55% shade when solar radiation measured outside the greenhouse exceeded $800 \text{ W}\cdot\text{m}^{-2}$. The daily light integrals were 18.5 ± 5.2 and $15.8 \pm 4.7 \text{ mol}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ in June and July, respectively, whereas the average daily temperatures were 24.7 ± 1.0 and $25.0 \pm 0.8^\circ\text{C}$ in June and July, respectively. For both experiments, all open petunia flowers were removed the day before the final spray application to allow for harvesting of freshly open flowers for the experiment.

Effect of CaCl_2 sprays (Expt. 1). Calcium chloride (anhydrous 96% purity; Thermo Fisher Scientific, Waltham, MA) was dissolved in deionized water to provide 0, 400, 800, or $1200 \text{ mg}\cdot\text{L}^{-1}$ Ca treatments. Spray applications were made between 1600 and 1700 HR and were applied at a rate of $204 \text{ mL}\cdot\text{m}^{-2}$ using hand sprayers. The CaCl_2 applications were made twice per week for 2 weeks. A fungicide control contained two active ingredients, cyprodinil 37.5% and fludioxonil 25% (Switch; Syngenta, Greensboro, NC), applied at the recommended rate ($449 \text{ mg}\cdot\text{L}^{-1}$) for ornamentals. The fungicide spray applications were made twice a week for 2 weeks from 1600 to 1700 HR. Five plants per treatment were treated with each of the Ca rates and the fungicide treatment, whereas 10 plants were treated with deionized water and later were divided into two control groups: noninoculated and inoculated with a conidial suspension. Eighteen flowers were harvested per treatment, and the individual flowers were considered as the individual experimental units.

Separating the effect of Ca, Cl, and EC (Expt. 2). Calcium chloride and KCl (99% purity, Thermo Fisher Scientific, Waltham, MA) were mixed in deionized water to provide EC treatments of 3.0 and $6.0 \text{ mS}\cdot\text{cm}^{-1}$ for each salt. The $3.0\text{-mS}\cdot\text{cm}^{-1}$ CaCl_2 solution provided $800 \text{ mg}\cdot\text{L}^{-1}$ Ca and $1420 \text{ mg}\cdot\text{L}^{-1}$ Cl, whereas the $6.0 \text{ mS}\cdot\text{cm}^{-1}$ solution provided $1600 \text{ mg}\cdot\text{L}^{-1}$ Ca and $2840 \text{ mg}\cdot\text{L}^{-1}$ Cl (Table 1). The $3.0\text{-mS}\cdot\text{cm}^{-1}$ KCl solution provided $960 \text{ mg}\cdot\text{L}^{-1}$ K and $871 \text{ mg}\cdot\text{L}^{-1}$ Cl, whereas the $6.0 \text{ mS}\cdot\text{cm}^{-1}$ solution provided $1920 \text{ mg}\cdot\text{L}^{-1}$ K and $1743 \text{ mg}\cdot\text{L}^{-1}$ Cl. Two additional KCl solutions were mixed in deionized water to provide the same Cl concentrations as delivered in the CaCl_2 solutions (e.g., 1420 and $2840 \text{ mg}\cdot\text{L}^{-1}$ Cl). The ECs of these two KCl solutions were measured at 4.2 and $8.2 \text{ mS}\cdot\text{cm}^{-1}$, respectively. Five plants were treated with each of the CaCl_2 and KCl applications, whereas 10 plants were treated with deionized water and were later divided into two control groups: noninoculated and inoculated with a conidial suspension. Eighteen flowers were harvested per treatment, and the individual flowers were considered as the individual experimental units.

Botrytis inoculation and evaluation. One day after the last treatment spray application, four to five newly open flowers per plant with 3 cm of pedicel were harvested between 1900

Table 1. Evaluation of botrytis blight severity on petunia flowers treated with calcium chloride (CaCl₂) or potassium chloride (KCl) spray applications and then inoculated with a conidial suspension of *Botrytis*. The KCl treatments provided equivalent concentrations of chloride (Cl) or electrical conductivity (EC) compared with the CaCl₂ treatments. Botrytis blight severity is expressed as a sum of the area under the disease progress curve (AUDPC).

Treatment	EC (dS·m ⁻¹)	Ca (mg·L ⁻¹)	K (mg·L ⁻¹)	Cl (mg·L ⁻¹)	Botrytis blight severity (AUDPC)
Control	0	0	0	0	305 a ^c
CaCl ₂	3.0	800	0	1,420	167 b
	6.0	1,600	0	2,840	73 c
KCl	3.0	0	960	871	320 a
	6.0	0	1,920	1,743	304 a
	4.2	0	1,565	1,420	301 a
	8.2	0	3,129	2,840	313 a

^cLetters indicate significantly different responses using the least significant difference test ($\alpha = 0.05$).

Fig. 1. Evaluation of botrytis blight severity on petunia flowers treated with calcium chloride spray applications or a fungicide (active ingredients: cyprodinil, 37.5%; fludioxonil, 25%) and then inoculated with a conidial suspension, with the exception of the uninoculated control group. Botrytis blight severity is expressed as a sum of the area under the disease progress curve (AUDPC). Letters indicate significantly different responses between treatments using the least significant difference test ($\alpha = 0.05$). Error bars represent ± 1 SE.

to 2000 HR. Flowers were placed immediately in 9-mL vials filled with 9 mL deionized water. These flowers were placed into 32.5 × 15.0 × 17.5-cm humidity chambers (BioTransport Carrier; Nalgene Corporation, Rochester, NY). Three humidity chambers were used per treatment, with six flowers per chamber. Each chamber contained a piece of polystyrene foam with holes in which the vials were inserted to be held upright. Water (500 mL) was placed in the bottom of each chamber to provide a high relative humidity (99.9%) as measured with a psychrometer (RH300; Extech Instruments, Nashua, NH). Before the chamber lids were sealed, the flowers were inoculated with hand sprayers providing 1 mL of 1×10^4 inoculum solution per flower and incubated for 72 h at 22 °C. Disease progression data were collected every 12 h by taking digital images of the flowers and rating the individual flowers in the images blindly at the end of each experiment. Infection severity was rated on a 1- to 9-point scale based on the area of infected corolla (1 = no infection; 9 = complete necrosis).

Data analysis. Data analysis was performed using JMP Pro version 13.2.0 (SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) was used to determine treatment effects and Fisher's least significant difference (LSD) test was used to compare means between treatments at $P < 0.05$. Treatments were analyzed using the area under the disease progression curve

(AUDPC) to evaluate the total reduction of botrytis blight severity for each treatment compared with the control. To calculate the AUDPC for each treatment, the following equation was used:

$$\text{AUDPC} = \sum_{i=1}^n \left[t \left(\frac{y_i - y_{\text{LAG}}}{2} \right) \right] + [t(y_{\text{LAG}} - 1)],$$

where n equals the number of time intervals to be summed, t is the time interval between each evaluation (12 h), y is the severity rating, and y_{LAG} is the previous severity rating time. The AUDPCs at each of the six time intervals are summed to calculate the total area under the curve.

For Expt. 1, the data set consisted of a 2×7 factorial model evaluating the two replications and seven spray treatments. The magnitude of the AUDPC response differed among the two replications, but the response to the spray treatments was similar within each replication, so data from the two replications were combined. Fisher's LSD test was used to compare means for the factor levels at $P < 0.05$. For Expt. 2, the data set consisted of the single factor of spray treatment to evaluate as replications were combined because they were not significantly different. A one-way ANOVA was performed to analyze the effect of spray treatment on reduction of botrytis blight severity. Fisher's LSD test was used to compare means for the factor level at $P < 0.05$.

Results

In Expt. 1, botrytis blight severity decreased as the Ca concentration supplied in the CaCl₂ solution increased from 0 to 1200 mg·L⁻¹ Ca (Fig. 1). All three CaCl₂ treatments provided better botrytis blight control than the fungicide treatment, which was not different from the inoculated control (i.e., the fungicide had no effect on *B. cinerea* infection). The 800-mg·L⁻¹ Ca treatment was statistically the same as the noninoculated control, whereas the 1200-mg·L⁻¹ Ca treatment had a lower botrytis blight severity than the noninoculated control.

In Expt. 2, botrytis blight severity decreased as the Ca application rate increased (Table 1). The KCl treatments that provided an equivalent solution EC or an equivalent concentration of Cl compared with the CaCl₂ treatments showed no significant differences when compared with the inoculated control, nor were there any differences among the replications. Therefore, this experiment indicates Ca is solely responsible for the reduction in botrytis blight severity.

Discussion

Two mechanisms are suggested to explain the effectiveness of Ca to reduce *B. cinerea* infection in horticultural crops. First, Ca binds with pectic material in the middle lamella to stabilize the cell wall, which makes it more difficult for fungal penetration into the tissue (Conway and Sams, 1984). Tomato fruit sprayed with Ca resulted in significantly increased levels of membrane and cell wall-bound Ca, as well as an increase in free Ca within the fruit, which also demonstrated a reduction of blossom end rot (Schmitz-Eiberger et al., 2002). Second, the binding of Ca to pectin in the middle lamella renders *B. cinerea* incapable of using pectin as a source of carbon, causing a reduction in polygalacturonase activity (Volpin and Elad, 1991). Volpin and Elad (1991) observed complete inhibition of polygalacturonase activity in liquid culture containing 120 mg·L⁻¹ Ca. Nigro et al. (2006) showed that multiple salts reduced polygalacturonase activity, but CaCl₂ demonstrated the greatest efficacy.

Botrytis cinerea exhibits incredible genetic plasticity, which allows for fungicide resistance to occur rapidly when under selection pressure from fungicide applications (Williamson et al., 2007). The results presented in this manuscript

showed inadequate control of *B. cinerea* from the fungicide treatment. Further testing of this isolate, which was recovered from a shipment of petunia plants received from a commercial grower, resulted in the discovery of the first isolate from greenhouse-grown ornamentals displaying resistance to six chemical classes of fungicides commonly used for botrytis blight management (Samarakoon et al., 2017b). Ca sprays are useful for managing botrytis blight, especially when dealing with fungicide-resistant isolates.

In conclusion, the results from this study clearly demonstrate Ca is the factor influencing the reduction in botrytis blight severity. Increasing the Ca concentration in the spray solution provides decreased botrytis blight severity up to 1200 mg·L⁻¹. This study demonstrates the potential usefulness of implementing Ca sprays during greenhouse production to reduce botrytis blight during shipping. Calcium spray applications provide another management strategy for growers to use in addition to conventional fungicides or as option to reduce the number of fungicide applications and thus reduce the selection pressure for fungicide resistance.

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