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The Effects of Aminoethoxyvinylglycine on Fruit Set in Emasculated, Hand-Pollinated Sweet Cherry *(Prunus avium L.)*

Per H. McCord[1] and Rainier W. Peters

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Abstract

Fruit set of sweet cherry is low when utilizing emasculated, hand-pollinated blossoms, as part of a breeding program. The ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) has been shown to increase fruit set in sweet cherry and has been approved for commercial use for this purpose. We investigated the ability of AVG to increase fruit set in emasculated, hand-pollinated blossoms over two crossing seasons. AVG was applied to blossoms either the day before or the day of emasculation, and the blossoms were pollinated the day after emasculation. Fruit set was evaluated when the cherries were still green, but after the natural abscission ('June drop') of unsuccessfully fertilized blossoms had occurred. Mixed model analysis showed no significant treatment effect on fruit set, but a significant cross effect was observed. Comparison of treatment effects within individual crosses was performed using Fisher's exact test. A wide range of effect was observed, including a negative effect on fruit set in several crosses when treated with AVG the day of emasculation, especially in 2019. When a positive effect of AVG was observed for a given cross, treatment the day before emasculation was more effective on fruit set than treatment the day of emasculation. In general, treatments with AVG produced a more positive effect in 2020 when more freeze damage occurred. AVG may have inhibited senescence of partially damaged blossoms, allowing fertilization and fruit set to occur. In summary, AVG can be used to increase fruit set in emasculated, hand-pollinated sweet cherry crosses, but it must be evaluated on a cross-by-cross basis, and the data suggest a greater effect when applied one day before emasculation.

Over the past twenty years, the worldwide production of sweet cherries has grown substantially (FAO, 2018). The United States of America is the second largest producer of sweet cherries in the world, having produced 321,400 t in 2019 (USDA, 2020). Within the United States, Washington is the largest producer of sweet cherries with a production of 216,800 t in 2019 (USDA, 2020). A critical component of a successful sweet cherry industry is the development of new superior cultivars. Washington State University's Irrigated Agriculture Research and Extension Center at Prosser has been a center of sweet cherry breeding for decades, and has released a number of important cultivars, such as 'Benton' (Olmstead et al., 2011), 'Chelan' (Lang et al., 1997), the standard early-ripening sweet cherry, and 'Tieton' (Olmstead et al., 2000). 'Rainier', the standard blush sweet cherry, was also developed at Prosser by the USDA.

The cherry breeding cycle starts in spring with crossing. Although large numbers of seed can be collected from desirable open-pollinated mother trees, the identity of the male parent is unknown, which limits genetic gain. Controlled bi-parental crosses can be made using bees inside screened cages, or by emasculation and hand-pollination. Emasculation and hand-pollination (EHP) gives maximum flexibility (i.e. one mother tree can be used for several crosses), but fruit set for such crosses is very low (Hedhly et al., 2009). In the breeding program at WSU, it is estimated that only around 10% of the

Washington State University, Irrigated Agriculture Research and Extension Center, 24106 N. Bunn Road, Prosser, Washington, 99350, USA.
[1] Corresponding author. Email address: phmccord@wsu.edu

blossoms in EHP crosses set fruit. Naturally, there is interest in treatments to improve fruit set in EHP crosses. One potential treatment is to apply an ethylene synthesis inhibitor. Ethylene is a plant growth regulator (PGR) responsible for a wide range of effects, such as bud and blossom drop, and blossom senescence (Serek at al., 1994). In sweet cherry, ethylene levels were higher at time of blossom abscission, and blossom abscission has been induced by ethylene accumulation (Blanpied, 1972). Research shows that early ovule degeneration is partly responsible for reduced fruit set in sweet cherry (Hedhly et al., 2007). Ethylene could induce ovule senescence and therefore shorten the window for fertilization of the cherry blossom. By treating blossoms with an ethylene inhibitor, it may be possible to keep the ovule and stigma viable for a longer period of time, thus improving the chances of fertilization (Racsko and Miller, 2012; Warner, 2014). Amino-ethoxyvinylglycine (AVG) is an ethylene inhibitor based on a naturally occurring fermentation product that blocks ethylene production in plants (Valent BioSciences, 2001). In a commercial formulation, AVG (trade name ReTain®) improved fruit set in sweet cherry, including 'Bing' (Valent Biosciences, 2019), 'Regina' (Racsko and Miller, 2012) and '0900 Ziraat' (Aglar et al., 2014), as well as in sour cherry (*P. cerasus* L.) 'Balaton' (Rothwell and Powers, 2019). The purpose of this study was to test the effects of AVG on fruit set in EHP sweet cherry crosses in the context of a breeding program.

Materials and Methods

The experiment was repeated over two crossing seasons (2019 and 2020) in the Roza research orchard at Washington State University's Irrigated Agriculture Research and Extension Center near Prosser, WA . Flower buds from each cross were divided into three treatment groups. Two of the groups received an application of AVG, and the third was a non-treated control. The solution was applied to the blossoms at a concentration of 880 mg/L ReTain powder. It was applied to the flower buds using a hand spray bottle to the point of runoff. The first treatment (AVG-2) was applied two days before pollination, while the second treatment (AVG-1) was applied one day before pollination. The flowers were emasculated the day before pollination, at approximately stages 56 to 57 of development (Herrero et al., 2017). For flowers receiving an AVG treatment on that day, emasculation was performed prior to spraying. Flowers were pollinated by hand using a small makeup applicator which consisted of a thin plastic rod tipped with a fine short-bristled brush. The brush was dusted with collected pollen stored in glass vials, and applied to the stigma. Viable fruits were counted after the natural abscission ('June drop') had occurred. The number of crosses evaluated per treatment each year are shown in Table 1.

Fruit set was calculated first as a percentage. These percentages were evaluated by a mixed model considering treatment as a fixed effect, and cross, year, and treatment × year as random effects. Cross and year were considered random effects because we intended to apply inferences across all levels of these variables, and not just those included in the study. The treatment × year interaction was considered random because the main effect of year was also considered

Table 1. The numbers of crosses and average number of blossoms per treatment involved in two years of fruit set experiments.

Year	Control	AVG-2	AVG-1
2019	14 ($\bar{x}$ =145)	6 ($\bar{x}$ = 195)	12 ($\bar{x}$ = 190)
2020	9 ($\bar{x}$ =123)	8 ($\bar{x}$ = 62)	9 ($\bar{x}$ = 106)

Table 2. Average and range of fruit set (%) for the control and AVG treatments over two crossing seasons (2019 and 2020). The number of fruits counted and the total number of flowers pollinated per treatment are listed in parentheses.

Year	Treatment	Average fruit set (%)	Range (%)
2019	Control	10.3 (199/1923)	0-47.5
"	AVG-2	11.8 (153/1292)	1.6-33.5
"	AVG-1	5.4 (128/2380)	0-25.8
2020	Control	8.9 (98/1104)	0-31.7
"	AVG-2	16.6 (83/500)	2.1-37.7
"	AVG-1	14.3 (136/953)	2.9-31.3

random. Additional models were explored considering the effects of females and/or males in place of cross, with both parental effects considered as random. Crosses were nested within years, and were not repeated in a given year. Because treatment × cross effects could not be tested directly, Fisher's exact test was performed on each cross separately, comparing counts of successful (fruit set) and unsuccessful fertilization of the control blossoms with either AVG-1 or AVG-2. The P-values for the individual tests were adjusted for multiple comparisons using the Benjamini-Hochberg procedure, which controls the false discovery rate (FDR). All the analyses were carried out using R statistical software (R Foundation for Statistical Computing, Vienna, Austria). Historical weather data was obtained from Washington State University's Ag Weathernet (www.weather.wsu.edu) from the weather station located at the research orchard.

Results and Discussion

Fruit set data for each treatment is shown in Table 2. In general, fruit set was low in the control groups. In 2019, fruit set averaged 10.3% across all control groups, while it 2020 control fruit set averaged 8.9%. There was also considerable variation for fruit set in the control groups. In 2019, fruit set ranged from zero (of which there were four crosses) to 47.5%. In 2020, control fruit set ranged from zero (two crosses) to 31.7%. Zhang et al. (2018) evaluated fruit set in emasculated flowers of four sweet cherry cultivars. Depending on cultivar and timing of pollination (days post-anthesis), fruit set ranged from zero to 67.1%. Fruit set of non-emasculated, open pollinated flowers was not measured in this study. However, Choi and Andersen (2001) reported average fruit set of 52.9% in a group of seven open-pollinated self-fertile sweet cherry genotypes. For non-self-fertile sweet cherries, Hedhly et al. (2009) focused on hand-pollinated crosses, and observed a two to three-fold decrease in fruit set between emasculated and non-emasculated flowers. For the AVG-2 treatment in 2019, fruit set was 11.8% overall while for AVG-1 the overall fruit set was 5.4%. In 2020, overall fruit set for AVG-2 was 16.6%, and the AVG-1 treatment resulted in overall fruit set of 14.3%. As with the control group, there was a wide range in % fruit set in both treatment groups over both years (Table 2).

The mixed model analysis suggested no significant overall treatment effects (P=0.378). There were also no significant year or treatment × year effects, i.e. these random terms had zero or near zero variance (P=0, 0.0004 respectively). However, the variance explained by cross was greater than the residual variance (0.0063 vs. 0.0059, respectively), and when measured by a likelihood ratio test, the effect of cross on fruit set was significant (P = 0.0005). When the cross effect was broken down into the ran-

dom effects of females and males, females contributed greater variance (0.004946 vs. 0.00179). The influence of temperature at time of AVG application was also explored, but no significant effect was found (data not shown). When testing treatment effects in individual crosses, the results varied considerably (Table 3). In 2019, the AVG-1 treatment resulted in significantly decreased fruit set in four crosses (PSC2019-024, -025, -058, and -062). AVG-2 had no significant effects on any crosses at $P < 0.05$. In 2020, AVG-1 significantly increased fruit set in three crosses (PSC2020-008, -019, and -042), and decreased fruit set in one cross (PSC2020-034). The AVG-2 treatment showed increased fruit set in the same 2020 crosses as AVG-1, plus PSC2020-021, and did not result in a decrease of fruit set in any 2020 crosses.

The effect of the mother tree on the effects of treatments designed to increase fruit set has been reported previously. Goldwin and Webster (1983) tested four different growth regulators over five years on five different sweet cherry cultivars. Treatments included gibberellic acid (GA_3), 1,3-diphenylurea (DPU) plus 2-naphthoxyacetic acid (NOXA), 1-naphthaleneacetic acid (NAA) or 2-2,4,5-trichlorophenoxy propionic acid (2,4,5-TP). 'Napoleon', 'Early Rivers', and 'Merton Glory' consistently and positively responded to the treatments, while 'Hedelfinger' showed a variable response, and there was no response in 'Merton Bigarreau' with DPU/NOXA or NAA. Webster et al. (2006) treated 'Stella' and 'Colney' trees with GA_3, GA_3 combined with NAA, or AVG. 'Stella' did not show increased fruit set with either treatment, whereas 'Colney' showed a strong response, particularly to the combined GA_3/NAA treatment.

Although the mixed model analysis suggested no significant year effects, the AVG treatments clearly had a more beneficial effect in 2020 based on the results of Fisher's exact tests of individual crosses. More flower bud damage was observed in 2020. This was most likely due to significant freeze events that occurred in late March and early April 2020, particularly on 26 March (-3.5° C) and 3 April (-4.5° C); similar frosts did not occur during the same period in 2019 (Table 4). Although blossoms with clearly dead pistils were not utilized in the study, it is possible that damage not observed by the naked eye was present. By retarding or preventing the senescence of damaged flowers, the AVG treatments could have improved fruit set. The negative effect of AVG on some crosses is curious. Decreased fruit set was only observed in AVG-1 treatments. It appears that for at least some crosses, AVG has a short-acting negative effect on fruit set, perhaps by inhibiting pollen germination on the stigmatic surface. In addition, as reviewed in An et al. (2020), the degradation of transmission tissues in the style promoted by ethylene may actually provide nutrition to and greater physical space for the elongating pollen tube. It is possible that the mother trees used in these crosses produce lower than average levels of ethylene. If ethylene biosynthesis were completely stopped in stylar tissue, it could also inhibit the growth of the pollen tube and prevent fertilization. Finally, if the beneficial effects of AVG are most apparent in the ovule itself, several days may be required for the uptake/transport of AVG to the ovule. This hypothesis is strengthened by the observation that in those crosses where AVG had a positive effect on fruit set, the AVG-2 treatment had a greater effect than AVG-1. Furthermore, an additional 2019 cross, PSC2019-030, was inadvertently not emasculated until 2 days after AVG application, (i.e. 3 days before pollination), and was the only cross that year where an AVG treatment showed a benefical effect (Table 3).

Whilst making the crosses it was observed that some stigmas had more of an exudate than others. No notes were taken during the experiment, but this could explain the effect of cross (or mother tree) on fruit set. In almond (*P. dulcis* (Mill.) D.A. Webb), Yi et al. (2006) observed better stigmatic receptivity in older blossoms, which was associated in

Table 3. List of crosses evaluated during the experiment, with counts of successes (fruits) and failures (pollinated, unfertilized blossoms), and the results of Fisher's exact test on treatment effects in individual crosses. The S-alleles for each parent (if known) are listed in parentheses. P-values were adjusted using the Benjamini-Hochberg method, which controls the false discovery rate.

Cross	Female	Male	Control	AVG-2	AVG-1	AVG-2 vs. control	AVG-1 vs. control
PSC2019-001	'Early Star' (S4'[y]S9)	'R19' (S4'S9)	21/186	--	8/57	NA[z]	0.4605
PSC2019-002	'FR09T084' (S4S9)	'R19' (S4'S9)	0/134	--	1/139	NA	0.5768
PSC2019-003	'FR09T086' (S3S4')	'R19' (S4'S9)	7/145	--	15/199	NA	0.3158
PSC2019-024	'Skeena' (S1S4')	'R14' (S3S9)	22/115	27/203	2/203	0.2298	<0.0001 (-)[x]
PSC2019-025	'Sunset Bing' (S3S4)	'R6' (S4'S9)	10/52	54/150	15/282	0.1358	0.0134 (-)
PSC2019-030	'Moby Dick' (--)	'DD' (S1S9)	5/50	25/49[w]	--	0.0077	NA
PSC2019-038	'Black Tartarian' (S1S2)	'FR09T086' (S3S4')	0/106	4/178	0/252	0.2298	1
PSC2019-051	'Sandra Rose' (S3S4')	'AL044' (--)	0/289	4/207	0/221	0.0780	1
PSC2019-057	'FR08T054' (--)	'R6' (S4'S9)	0/193	3/179	0/138	0.1983	1
PSC2019-058	'FR23T115' (S3S9)	'DD' (S1S9)	29/32	54/107	32/192	0.0863 (-)	0.0100 (-)
PSC2019-062	'FR02T068' (S4'S4')	'R29' (S1S4')	29/146	--	3/160	NA	<0.0001 (-)
PSC2020-008	'Schmidt' (S2S4)	'R29' (S1S4')	0/117	9/46	7/79	0.0002	0.008

PSC2020-009	'FR03T002' (S3S4')	'DD' (S1S9)	11/126	1/47	16/95	0.2215	0.1583
PSC2020-010	'FR11T019' (S3S4)	'FR02T068' (S4'S4')	6/89	2/28	12/80	0.6724	0.1740
PSC2020-019	'FR09T086' (S3S4')	'R3' (S1-)	26/148	99/142	67/147	<0.0001	0.0006
PSC2020-020	'FR08T054' (S2S3)	'R6' (S4'S9)	10/191	11/118	5/130	0.2653	0.4605
PSC2020-021	'FR35T101' (S5-)	'Black Pearl' (S4S13)	18/132	16/45	12/118	0.0304	0.3775
PSC2020-033	'FR01T039' (S3S4')	'DD' (S1S9)	13/28	11/32	8/47	0.4401	0.0863 (-)
PSC2020-034	'Spalding' (S2S4)	'CR18T35' (--)	14/87	4/52	3/99	0.2298	0.0131 (-)
PSC2020-042	'Sam' (S2S4)	'R6' (S4'S9)	0/88	29/48	6/22	<0.0001	0.0006

[z] One or the other treatment not applied. [y] Self-compatible mutant of S4 (universal pollen donor). [x] Negative effect on fruit set. [w] AVG applied 3 days before pollination. This treatment was not included in the mixed model analyses.

Table 4. Frost dates and minimum temperatures at the WSU Roza orchard for the period 15 March-15 April 2019 and 2020.

2019		2020	
Date	**Minimum Temperature °C**	**Date**	**Minimum Temperature °C**
15 March	-2.6	15 March	-3.2
16 March	-1.8	16 March	-2.3
17 March	-0.5	17 March	-3.9
18 March	-0.7	18 March	-1.0
15 April	-0.5	19 March	-0.7
		25 March	-1.5
		26 March	-3.5
		1 April	-0.4
		2 April	-2.5
		3 April	-4.5
		4 April	-2.0

part with increased amounts of exudate. In apricot (*P. armeniaca* L.), the stigma is not yet mature at the time of blossom opening, and stigmatic receptivity appears to be greatest 2 days after bloom (Egea and Burgos, 1992). This is similar to the findings of Zhang at et al. (2018), who found that pollen germination in sweet cherry was highest within 2 days of bloom. The timing of emasculation is critical to discourage insect pollination, but it could be of benefit to wait an additional 1 or 2 days before hand pollinating.

The classical first step in a plant breeding program is crossing desired parents in an effort to produce progeny with superior allelic combinations. To obtain accurate information about cross performance and to increase the probability of identifying superior offspring, reasonable numbers of seed per cross are needed. In species such as sweet cherry that only produce one seed per fruit, maximizing fruit set is crucial to generating sufficient progeny for meaningful selection to occur. We have shown that the effects of AVG on fruit set are complex and are cross and likely time dependent. For highly valuable crosses that are known to have limited fruit set, AVG application the day before emasculation (2 days prior to pollination) may result in sufficient numbers of seed for reliable evaluation. Due to the effort and expense in generating seed in breeding programs, additional research on improving fruit set in EHP crosses is warranted. In addition to exploring the effects of blossom age described earlier, areas of research should include variable rates of AVG, timing of AVG application (more than 2 days before pollination), as well as testing of other plant growth regulators such as GA_3/NAA and DPU/NOXA. Progress in breeding superior cultivars will be accelerated as larger families of desirable crosses can be more reliably produced.

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