Effect of Fruiting and Biennial Bearing Potential on Spur Quality and Leaf Gas Exchange in Apple

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ABSTRACT. Uniform annual apple (*Malus ×domestica*) fruit production is highly dependent on consistent flower formation from year to year, as inconsistent flowering can lead to the biennial bearing observed in some high-value cultivars. The presence of fruit on a spur has been considered the main cause of the expression of biennial bearing and the inhibition of flower initiation, with a number of theories being introduced to explain the phenomenon. In the current experiment, individual spurs of annual bearing cultivars (Gala, Ruby Jon, and Pink Lady) and biennial bearing cultivars (Honeycrisp, Fuji, and Golden Delicious) were thinned to a single fruit or completely defruited at petal fall. Spurs were sampled at the end of the growing season. Effects of fruiting on spur characteristics such as spur and bourse leaf area, stomatal density, leaf gas exchange, and flower formation were determined. Across all cultivars, the presence of fruit on a spur did not affect spur characteristics or flower formation compared with nonfruiting spurs. Similarly, flowering was unaffected by those factors associated with greater spur carbohydrate status, such as bourse leaf area and assimilation rate. Cultivars with greater transpiration and stomatal conductance (g_s) rates had lower rates of flower formation. Future studies should focus on xylem flow and expression of genes regulating flowering and plant growth regulators in annual and biennial bearing cultivars.

One of the most challenging problems associated with fruit tree production is biennial bearing. Biennial bearing is “the fluctuation in cropping from year-to-year caused by irregular flowering” (Hirst, 2017). Considerable research has been conducted to investigate biennial bearing; however, it remains a complicated problem (Bangerth, 2009; Elsysy and Hirst, 2017; Monselise and Goldschmidt, 1982). A number of high-value apple cultivars exhibit biennial bearing, including Cameo, Fuji, and Honeycrisp. Biennial bearing is affected by a number of factors such as growth and development processes, plant growth regulators, carbohydrate content, and fruiting (Greene, 1996; Singh, 1948). These factors may interact at both the spur and whole-tree levels. Furthermore, fruit, and especially seeds within the fruit, appeared to be the major stimuli to biennial bearing (Chan and Cain, 1967; Dennis, 1967). Seeds may increase the demand by fruit for resources such as nutrients, plant growth regulators, and carbohydrates (Heinicke, 1917). This demand by the fruit for resources within the spurs could affect spur quality and flower formation.

Previously, spur quality has been measured in terms of leaf area, spur leaf number, leaf dry weight, specific leaf weight, and mean leaf size (Barritt et al., 1987; Ferree, 1989). Total spur leaf area varies among cultivars and relates to tree productivity and efficiency (Rom and Ferree, 1984). Leaf area per spur has a quantitative relationship with flower formation (Rom and Barritt, 1990), and spur density is correlated with yield efficiency (Warrington et al., 1990). Spur characteristics are impacted by rootstocks (Hirst and Ferree, 1995a; Warrington et al., 1990), and spur quality was improved by the application of triazole compounds (Ferree and Schmid, 1987). Hence, spur morphological and anatomical attributes may be a way to study the effects of fruiting on spur quality and flower formation.

Four main genomic regions, containing floral genes, gibberellin oxidase genes, and meristem identity genes, associated with biennial bearing were identified in the genome of apple using quantitative trait locus analysis (Guitton et al., 2012). Homologs of the floral promoter *Flowering Locus T* and the floral inhibitor *Terminal Flower 1* were characterized in several fruit species including apple (Kotoda and Wada, 2005; Kotoda et al., 2006; Mimida et al., 2013). Plant growth regulators have been suggested to play a key role in bud development and flower formation in apple (Xing et al., 2015).

Leaves play several roles; they provide carbohydrates and plant growth regulators, and work as the main receptors for

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Table 1. Apple trees growing in Lafayette, IN, used in this study of the effect of fruiting and biennial bearing potential on spur quality and leaf gas exchange in apple. Bearing classification was based on experience with these cultivars.

Cultivar	Yr planted	Rootstock	Bearing classification
Fuji	2001	B.9	Biennial
Gala	2001	B.9	Annual
Golden Delicious	2000	G.16	Biennial
Honeycrisp	2003	M.7	Biennial
Pink Lady	1999	M.9 EMLA	Annual
Ruby Jon	2001	B.9	Annual

Fig. 1. (A) Apple fruiting spur showing spur leaves emerging directly from the spur, and bourse leaves emerging from the bourse shoot. (B) Leaves on 1-year-old shoot.

environmental stimuli (Hanke et al., 2007). In addition, leaves transpire and affect the flow of substances through the xylem stream. In temperate deciduous trees, stomatal density correlates positively with transpiration and photosynthetic potential (Aasamaa et al., 2001; Sack et al., 2003, 2005). Moreover, stomatal density varies among apple cultivars (Cowart, 1936; Slack, 1974; Warrit et al., 1980). Fruiting affects several physiological processes in the leaves. For instance, in different fruit tree types, fruit-bearing trees showed increased CO₂ assimilation rates compared with nonfruit-bearing trees (Palmer et al., 1991; Syvertsen et al., 2003; Urban et al.,

Fig. 2. Gas exchange of 'Honeycrisp' apple leaves measured throughout the day ≈2 months after full bloom in Lafayette, IN. (A) CO₂ assimilation rate. (B) Stomatal conductance.

2004). However, no differences in CO₂ assimilation rates were observed in cherry (*Prunus avium*) and sweet orange (*Citrus sinensis*) between on and off crop years within the same cultivars (Monerri et al., 2011; Nebauer et al., 2013; Roper et al., 1988). A reduction in photoassimilate utilization was accompanied by the accumulation of nonstructural carbohydrates, especially starch, in 'Braeburn' apple leaves bearing low or no crop load (Wünsche et al., 2005). This reduction may have been induced by plant growth regulators or it may be a response to a change in intercellular CO₂ concentrations. Moreover, the availability of photoassimilates could possibly play a regulatory role in floral induction (Shalom et al., 2012).

The relationship between carbohydrate content and flower formation has been well defined for a number of crops. For example, fruit removal of 'Wiking' mandarin (*Citrus reticulata*) by midsummer led to an increase in starch content and flower formation (Goldschmidt and Golomb, 1982). In 'Murcott' mandarin, defruiting affected the expression of genes related to photosynthesis, flower formation genes, and flower formation (Shalom et al., 2014). Sugar has been suggested to be a florigenic substance in plants (Ortiz-Marchena et al., 2015; Wahl et al., 2013). In addition, the network of flower formation in apple was suggested to be mediated by the crosstalk between sugar and plant growth regulator signals (Xing et al., 2015).

The objective of our experiment was to compare spur quality and flower formation of single-fruited and defruited spurs of annual and biennial apple cultivars. We hypothesized that defruiting would have a greater stimulating effect on flowering of biennial bearing cultivars than annual bearing cultivars, and that spur quality attributes that lead to greater spur carbohydrate levels will be associated with greater levels of flowering.

Fig. 3. Total bourse leaf area as affected by cultivar and fruiting treatment at the end of the 2014 growing season. Analysis of variance within every cultivar showed no significant effects of fruiting on total bourse leaf area during Summer 2014 for any of the six apple cultivars.

(less than a 400-m spread). Growing conditions, including soil type, and tree management were similar for all trees. Within each cultivar, fruiting treatments were arranged as a completely randomized design. Four healthy trees of each cultivar were chosen and 120 flowering spurs were selected randomly at bloom across the four trees. At petal fall, 60 of these spurs were thinned to king flower only, whereas the other 60 selected spurs were completely defruited. All spurs were tagged using durable plastic tags identified by color and number. For each cultivar and treatment, 60 spurs from each cultivar and treatment were collected at the end of the growing season, 150 d after full bloom.

Measurements

Fig. 4. Stomatal density of spur, bourse, and shoot leaves of six apple cultivars measured toward the end of the 2014 growing season. No significant differences existed between defruited and fruited spurs, so data were pooled within each cultivar. Different letters represent significant differences ($P < 0.05$) among different leaf types within each cultivar.

ANATOMICAL MEASUREMENTS. The following measurements were made on sampled spurs: 1) spur and bourse leaf number and leaf area (LI-3000; LI-COR, Lincoln, NE), 2) stomatal density, and 3) flower formation [examined under a dissecting microscope (SZ-ST5; Olympus Corp., Center Valley, PA)] to determine flower formation for each bourse (Hirst and Ferree, 1995b).

STOMATAL DENSITY. Stomatal density of spur, bourse, and shoot leaves was measured from five single-fruited and five defruited spurs (Fig. 1). Abaxial epidermal anatomy was characterized on the second fully developed bourse and spur leaf. The abaxial epidermis adjacent to the leaf midrib was placed initially onto a cyanoacrylate droplet on a glass slide for 2 s to remove trichomes, then was placed on another cyanoacrylate droplet for 30 s to imprint the abaxial epidermis. Four images from different spots of each imprint were obtained from each slide under 200× magnification using a generic description microscope (OptiPhot2; Nikon, Tokyo, Japan) and microscope camera software MiniSee (Scopetek Electric Co., Hangzhou, China). In all calculations, stomata were considered to be a pair of fully developed guard cells. Stomata were counted using Image J software (National Institutes of Health, Bethesda, MD) and are expressed per square millimeter leaf area.

LEAF GAS EXCHANGE. Leaf gas exchange was measured on the third fully expanded, attached bourse leaf on three single-fruited and three defruited spurs in well-illuminated positions on the west side of the trees. Measurements were transpiration, g_s , internal CO_2 concentration, assimilation, and water use efficiency (WUE). All measurements were taken 66 d after full bloom on a sunny day of $\approx 24^\circ C$ between 0930 and 1430 HR. The optimal time of day was based on steady rates of assimilation and g_s found during a pilot study on 'Honeycrisp' trees by measuring the same leaf throughout the day (Fig. 2). Measurements were taken using a portable infrared gas analyzer system (CIRAS-3; PP Systems, Amesbury, MA) fitted with a 4.5-cm² leaf chamber with built-in light emitting diodes (LEDs; 475-, 528-, 625-, and 425–650-nm peak wavelengths for the blue, green, red, and white LEDs, respectively). The conditions were reference CO_2 concentration, 400 $\mu mol \cdot mol^{-1}$;

Materials and Methods

PLANT MATERIALS. This experiment was conducted in Spring 2014 on six apple cultivars, three generally considered to be annual bearing (Gala, Pink Lady, and Ruby Jon) and three generally considered to have greater biennial bearing potential (Fuji, Honeycrisp, and Golden Delicious) (Table 1). All cultivars were planted with 3 × 5 m spacing at the Samuel G. Meigs Horticulture Facility of Purdue University in Lafayette, IN.

This experiment used mature trees growing on dwarfing 'Budagovsky 9' (B.9) or semidwarfing ['Geneva 16' (G.16), 'Malling 9' (M.9 EMLA), and 'Malling 7 EMLA' (M.7)] rootstocks (Table 1). All trees chosen for this study appeared healthy and were 3 to 5 m tall regardless of rootstock. Trees were trained to a vertical axis tree form and were managed according to standard commercial practices. Crop load was adjusted manually by hand-thinning at petal fall to achieve a medium crop load of 120–140 fruit per tree to avoid possible effects of excessive crop load on leaf gas exchange (Nielsen et al., 2016).

EXPERIMENTAL PROCEDURE. All trees were located at the same experimental farm within close proximity to each other

Fig. 5. The effect of fruiting on flower formation in six apple cultivars during Summer 2014. Logistic regression analysis within each cultivar showed no significant effect of single fruiting on bourse flower formation compared with defruited spurs. Flower formation is presented as a proportion of flowering spurs within each treatment for each cultivar.

Fig. 6. Average seed number per fruit of annual and biennial apple cultivars growing in Lafayette, IN, during 2014.

leaf temperature, 25 °C; relative humidity, $\approx 60\%$; and flow rate through the chamber, 300 mL·min⁻¹. To determine the light-saturated rates of photosynthesis, g_s , transpiration, respiration, and WUE, the light unit in the cuvette was programmed to provide 1000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (33% red light, 33% blue light, and 34% green light). Measurements were recorded after reaching a steady state (≈ 90 s).

STATISTICAL ANALYSIS. Analysis of variance and mean separation of parameters between defruited and single-fruited spurs were performed for all data. However, a binomial logistic regression with mixed-effects model was used to test flower formation. All data analyses were performed using R software version 3.2.2 (14 Aug. 2015) “Fire Safety” in the R statistical package (R Foundation, Vienna, Austria).

Results

EFFECTS OF FRUITING ON ANATOMICAL AND MORPHOLOGICAL CHARACTERISTICS. Bourse leaf area was not different in fruiting

spurs compared with defruited spurs of the same cultivar (Fig. 3). There was also no relationship between the bearing potential of these cultivars and bourse leaf area. For example, both ‘Pink Lady’ and ‘Ruby Jon’ are characterized as annual bearing cultivars, but there was a trend for ‘Pink Lady’ to have the greatest bourse leaf area and for ‘Ruby Jon’ to have the lowest among all cultivars in this study (Fig. 3).

STOMATAL DENSITY. In all cultivars, the presence of fruit did not affect spur or bourse leaf stomatal density. Therefore, data from defruited and single-fruited spurs were combined. In all cultivars, shoot leaves had greater stomatal density than spur leaves, with bourse leaves tending to be intermediate (Fig. 4). There were no differences between biennial cultivars as a group and annual bearing cultivars.

FLOWER FORMATION. In all cultivars, there was no significant effect of fruiting on flower formation, although there was a trend in every cultivar for fruiting spurs to exhibit less flowering (Fig. 5). Biennial cultivars generally exhibited a decreased flowering proportion compared with annual cultivars. Fruit of both annual and biennial cultivars contained six to eight seeds per fruit, and no differences in seed number between annual and biennial cultivars was observed (Fig. 6). In addition, there was no effect of seed number on flower formation within any of the cultivars.

EFFECTS OF SINGLE FRUITING ON LEAF GAS EXCHANGE PARAMETERS.

The presence of fruit had no effect on leaf gas exchange in any of the studied cultivars (Table 2). Furthermore, WUE was unaffected by fruiting on single-fruited spurs compared with defruited spurs on the same trees (Elsysy and Hirst, unpublished data). However, both transpiration and g_s were greater in biennial cultivars compared with annual cultivars. Furthermore, analyses using Pearson correlation coefficients among different gas exchange processes revealed significant correlation coefficients ($P < 0.05$) between the following: 1) transpiration with g_s , internal CO₂ concentration, and assimilation; and 2) WUE with internal CO₂ concentration and stomatal density (Table 3). Significant correlation coefficients ($P < 0.05$) occurred between flower formation and both transpiration and g_s [$r = -0.61$ and -0.62 , respectively (Fig. 7)].

Discussion

Theories to explain the effect of fruiting on flower formation generally fall into three categories: 1) plant growth regulators (Guittou et al., 2012; Krasniqi et al., 2013; Pellerin et al., 2012),

Table 2. Effect of fruiting, bearing potential, and their interaction on gas exchange in six apple cultivars.

Bearing	Cultivar	Single fruited	Defruited	$\bar{X}$ cultivar	<i>P</i> value
Transpiration [H_2O ($\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)]					
Biennial	Honeycrisp	2.47	2.93	2.7	0.40
	Golden Delicious	3.26	3.06	3.16	0.82
	Fuji	2.77	2.47	2.62	0.33
	Biennial mean	2.83	2.82	2.83	
Annual	Ruby Jon	2.83	2.66	2.75	0.64
	Pink Lady	2.09	2.26	2.18	0.75
	Gala	2.24	2.26	2.25	0.96
	Annual mean	2.39	2.40	2.39	
	Fruiting (F)				0.98
	Bearing (B)				0.04**
	F $\times$ B				0.95
Carbon assimilation [CO_2 ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)]					
Biennial	Honeycrisp	15.67	20.27	17.97	0.14
	Golden Delicious	21.97	22.20	22.09	0.87
	Fuji	16.17	17.83	17	0.56
	Biennial mean	17.94	20.1	19.02	
Annual	Ruby Jon	18.87	19.17	19.02	0.89
	Pink Lady	19.77	18.40	19.09	0.59
	Gala	15.90	16.17	15.85	0.91
	Annual mean	17.88	17.91	17.9	
	F				0.38
	B				0.36
	F $\times$ B				0.26
Stomatal conductance [H_2O ($\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)]					
Biennial	Honeycrisp	263.3	396.0	329.65	0.40
	Golden Delicious	435.3	437.0	436.15	0.82
	Fuji	331.3	251.7	291.5	0.34
	Biennial mean	343.3	361.57	352.43	
Annual	Ruby Jon	326.3	291.3	308.8	0.68
	Pink Lady	252.3	269.0	260.65	0.75
	Gala	227.7	230.0	228.85	0.96
	Annual mean	268.77	263.43	266.1	
	F				0.90
	B				0.05*
	F $\times$ B				0.8

*Significant at $P \leq 0.05$.

Table 3. Correlation coefficients among leaf gas exchange, stomatal density, leaf area, and flower formation of six apple cultivars in Summer 2014.

	Flowering ^z	E	g_s	Ci	A	WUE	Stomatal density
E	-0.61*						
g_s	-0.62*	0.93***					
Ci	-0.51	0.59*	0.42				
A	-0.27	0.64*	0.78**	-0.15			
WUE	-0.36	-0.42	-0.15	-0.81***	0.39		
Stomatal density	-0.16	0.21	0.06	0.52	-0.31	-0.62*	
Leaf area	0.02	-0.37	-0.14	-0.20	0.05	0.56*	-0.33

^zFlowering = flower formation.

*, **, ***Significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

E = transpiration; g_s = stomatal conductance; Ci = internal CO_2 concentration; A = assimilation; WUE = water use efficiency.

2) source–sink competition (Bruchou and Génard, 1999; Palmer et al., 1991; Saa and Brown, 2014), and 3) the interaction between plant growth regulators and sink–source competition (Smith and Samach, 2013; Untiedt and Blanke, 2001).

sink–source relationship, 2) change in the balance of plant growth regulators, and 3) higher xylem flow in biennial cultivars compared with annual cultivars. Actually, the newly well-defined interaction among sugar, photoassimilates, and the expression level of flower formation genes in citrus (Shalom et al., 2014) and

In a related experiment on flower formation in ‘Honeycrisp’ apple, we found greater flower formation on single-fruited spurs compared with those bearing two fruit (M.A. Elsysis and P.M. Hirst, unpublished data). Hence, in the current study, we examined the effect of bearing a single fruit on spur quality and flower formation of annual and biennial cultivars. In contrast to our previous results with double-fruited spurs, in this experiment we found that single-fruited spurs showed similar spur characteristics and flower formation to nonfruiting spurs in both annual and biennial cultivars (Table 2; Figs. 3–5). Apparently, single fruiting did not stimulate the local inhibition effects previously suggested by plant growth regulators, source–sink competition, or their interaction.

Fruiting affects several leaf physiological processes (Palmer et al., 1991; Syvertsen et al., 2003; Urban et al., 2004). However, the combined effects of individual spur fruiting and cultivar bearing potentials on leaf anatomy and physiological processes have not been examined. In this study, we propose that annual and biennial cultivars possess different photoassimilation strategies, because both g_s and transpiration were greater in biennial cultivars compared with annual cultivars (Table 2; Fig. 7). Stomatal density was found previously to be correlated with transpiration in a transgenic apple species (Liao et al., 2017), although no such relationship was found in the current study (Table 3) and no differences in stomatal density were found among cultivars (Fig. 4). Transpiration was closely related to g_s but not stomatal density (Table 3). Hence, it would seem that differences in g_s and resultant transpiration rates among these cultivars, may influence flower formation (Fig. 7).

In addition, both g_s and transpiration correlated positively with assimilation (Table 3). Hence, we suggest that high g_s and transpiration rates in biennial cultivars may affect flower formation via 1) different consumption rates of photoassimilates in biennial cultivars that could affect the

Fig. 7. Relationship between flower formation and (A) transpiration and (B) stomatal conductance in six apple cultivars during Summer 2014. Flowering (measured as a percentage) represents the proportion of flower formation for each treatment in each cultivar.

apple (Xing et al., 2015) suggests that transpiration and g_s may influence flower formation by affecting photoassimilates or sugar levels. Differences found here in transpiration rates between biennial and annual bearing cultivars, and the relationship between transpiration rates and flowering, are likely the result of changes in plant growth regulators transported in the xylem, because no differences in assimilation were found (Table 2; Fig. 7).

In conclusion, this research shows that in both annual and biennial cultivars, bearing a single fruit per spur does not necessarily inhibit flower formation for the following year's crop when the overall tree crop load is not excessive. Based on the three biennial and three annual cultivars studied in this experiment, different bearing types appear to have different gas exchange characteristics, with biennial cultivars having greater g_s and transpiration rates. Further research on the effects of different transpiration and g_s rates on xylem flow, and the expression patterns of photosynthesis genes, plant growth regulator genes, and flowering genes in buds of annual and biennial cultivars could explain the complicated networks involved in apple flower formation.

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