Characterization of Stigma Exudates in Aqueous Extracts from Apple and Pear Flowers

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Abstract. **The stigmatic secretions of pomaceous flowers serve as a natural medium not only for pollen, but also for the pathogen *Erwinia amylovora* (Burr.) Winslow et al. and other microorganisms. To understand the microecology on the stigma, exudates from cultivars of pear (*Pyrus communis* L.), apple (*Malus pumila* P. Mill.), and crab apple [*Malus mandshurica* (Maxim.) Kom.] were analyzed for free sugars and free amino acids as available carbon and nitrogen sources. Extracts were obtained at different stages of anthesis by submerging and sonicating stigmas in water. Certain free sugars (glucose and fructose) and free amino acids (proline, asparagine, glutamic acid, and glutamine) were consistently predominant and increased during anthesis. Apple stigma extracts were also analyzed for polysaccharides and proteins. Of major components identified for apple, free sugars made up 4.5% by mass; polysaccharides (composed of arabinose and galactose), 49.6%; and proteins, 45.9%. The two largest components are likely present as glycoproteins. This may be the first report on characteristics of rosaceous stigma exudates that includes the identity of specific free sugars, free amino acids, and polysaccharide subcomponents. Discussion includes the comparison of pomaceous stigma exudates to those of other plants and the microecological implications.**

Given the importance of the stigma to fruit development in agriculturally prominent species in Rosaceae, there is surprisingly little information about the composition of stigma exudates of plants in this group. Although such data are extensive for a number of other flowering plants (Knox, 1984), studies involving rosaceous species are limited to histological investigations indicating the general presence of intercellular carbohydrates, proteins, and lipids in stigmatic tissue (Cresti et al., 1980; Stant, 1981; Uwate and Lin, 1981) and to the recent focus on S-RNases (Certal et al., 1999; Ishimizu et al., 1996; McClure and Franklin-Tong, 2006; Sonneveld et al., 2005). The deficiency of information for tree fruit species may be partly the result of the relatively small size of stigmas and brevity of the bloom period.

The stigma exudates of flowering plants serve as a natural medium for pollen germination and other functions in the pollen–stigma interaction (Heslop-Harrison, 2000;

Knox, 1984). Exudates produced by flower stigmas of pome fruit trees are also an excellent medium for many microorganisms (Pusey, 1997, 1999b; Stockwell et al., 1999). Pomaceous stigmas and their exudates not only sustain a wide diversity of bacteria, yeasts, and other fungi, but they also support microbial population densities far exceeding those of most other aerial plant surfaces. In a review by Johnson and Stockwell (1998), this point was illustrated by comparing maximum bacterial populations on bean leaves to those on pear stigmas. *Pseudomonas syringae* pv. *syringae* is reported to attain populations of ≈10[6] colony-forming units (cfu) per gram fresh weight of bean leaves (Beattie and Lindow, 1994), but the carrying capacity of bacteria on pear stigmas is estimated at 10[9] to 10[10] cfu/g. Another unique aspect of the stigma as a microbial habitat is its frequent contact with pollinating insects that carry microorganisms from flower to flower (Johnson et al., 1993; Thomson et al., 1992; Vanneste, 1996).

Microbial colonization on stigmas has been given considerable attention in connection with fire blight, a serious disease of apple, pear, and other rosaceous plants. The stigma is the primary site supporting establishment of the causal bacterium, *Erwinia amylovora* (Burr.) Winslow et al. (Johnson and Stockwell, 1998). Typically, epiphytic populations increase on stigmas during warm periods but usually do not cause disease until rain or dew facilitates movement of the

pathogen from the stigma to the hypanthium where infection occurs through nectarthodes (Thomson, 1986). *Erwinia amylovora* appears to be well adapted epiphytically to the stigmatic surfaces of even nondisease–host rosaceous species (Johnson et al., 2006). As an alternative to the use of antibiotics for suppression of *E. amylovora* on apple and pear blossoms, researchers have evaluated other naturally occurring microbial residents on flowers as potential biological control agents for fire blight (Johnson and Stockwell, 2000). Beneficial strains exhibiting antagonism toward *E. amylovora* suppress the pathogen by competing for space and nutrients and producing antibiotic compounds. In 1996, *Pseudomonas fluorescens* (Trevison) Migula strain A506 was the first microbial agent to become available commercially for fire blight management (Johnson and Stockwell, 2000). Others have since been developed, and in at least one case, farm managers are given the option of delivering the product to flowers using honeybees (Vanneste, 1996; Vanneste et al., 2002).

Knowledge of the biochemistry of stigma exudates is not only a prerequisite to understanding pollen–stigma interactions (Kandasamy and Vivekanandan, 1986; Knox, 1984), but it is necessary for understanding microbial interactions that may be advantageous for preventing fire blight of apple and pear. Chemical analyses of the pomaceous exudates provide basic information related to reproduction in an important plant group not yet given such attention and may reveal major nutrient sources supporting both disease-causing and beneficial microorganisms on stigmas. The latter objective was the primary motivation for the current study. Stigma exudates from apple and pear at different stages of anthesis were first analyzed for free sugars and free amino acids that may serve as available carbon and nitrogen sources for microbial activity. Further analyses were performed with apple to identify other major components of the exudates.

Materials and Methods

Extraction procedure. Stigma exudates were collected from flowers of pear (*Pyrus communis* L.) and apple (*Malus pumila* P. Mill.) in 2003 and 2004. Tree cultivars were 'Anjou' and 'Bartlett' pear and 'Fuji', 'Gala', and 'Golden Delicious' apple, all located in research orchards near Wenatchee, WA. Blossoms were not protected from indigenous microorganisms or insect visitors during the sampling period. Crab apple [*Malus mandshurica* (Maxim.) Kom.], maintained in a greenhouse and used routinely as a source of blossoms for laboratory assays (Pusey, 1997), was evaluated along with apple and pear cultivars in 2004. Flowers were sampled at three stages based on anther dehiscence: nondehisced, 40% to 60% dehisced, and 100% dehisced. For each cultivar and stage per year, a minimum of two groups of 50 flowers was sampled. Each
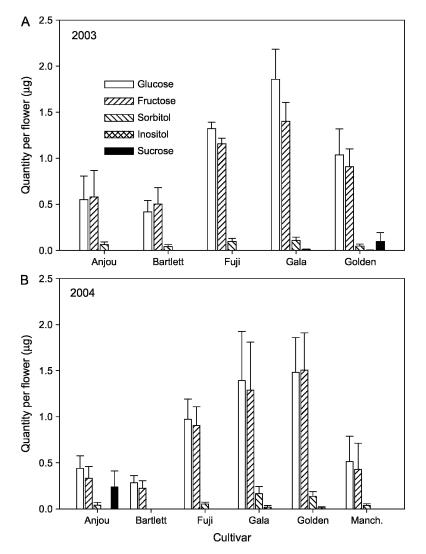
Fig. 1. Free sugars detected in stigma exudates from 'Anjou' and 'Bartlett' pear (*Pyrus communis*); 'Fuji', 'Gala', and 'Golden' apple (*Malus pumila*) and 'Manchurian' crab apple (*Malus mandshurica*) in (**A**) 2003 and (**B**) 2004. Each bar represents mean (±SE) of at least six samples, each derived from 50 flowers.

excised flower was temporarily maintained with cut pedicel supported in a 2-mL vial filled with water. The flowers were transported to the laboratory where either stigma exudates were collected the same day or flowers were held overnight at 1 or 2 °C before extracting exudates. Anthers were removed and stigma tips of 50 flowers were submerged, one flower at a time, in the same volume of 600 μL water held in a microfuge tube while sonicated for 10 s per flower. Pollen, debris, and microorganisms were removed from the aqueous solution by centrifugation at 16,000 g_n for 5 min and by subsequent filtration of supernatant in a syringe-driven unit with a PVDF membrane, 4-mm diameter and 0.22-μm pore size (Millex-GV; Millipore Corp., Billerica, MA). Macromolecules were eliminated by filtration in centrifugal devices, the first with a 100-kDa cutoff and a second one with a 3-kDa cutoff (Nanosep 100K and 3K Omega; Pall Corp., Ann Arbor, MI). Filtrates were stored at –20 °C until analyses were performed.

To substantiate how the free sugar composition of stigma exudates compares with nectar sugar composition, which has already been documented for apple and pear (Bieleski and Redgwell, 1980; Campbell et al., 1990; Wykes, 1952), flowers sampled in 2004 for stigma exudate analyses were also evaluated for nectar sugars. Nectar was collected from the hypanthium of each flower with a micropipet, and then pooled samples from each group of 50 flowers were analyzed for sugars using the procedure described below for stigma exudates.

Sugar analysis. Sugars were converted to trimethylsilyl derivatives before gas chromatography (GC) analysis using the method of Mateo et al. (1987) with the main modification involving preparation of keto groups with methoxyamine hydrochloride to prevent multiple derivatives from forming during silylation (Horning et al., 1968). To 150 μL of stigma sample, 20 μg (in 20 μL) of phenyl β-D-glucose was added as an internal standard, and the extract was concentrated to dryness with a vacuum evaporator (Savant

Speed Vac Concentrator; Savant Instruments Inc., Holbrook, NY). Afterward, 5 μL 2-dimethylaminoethanol and 125 μL methoxyamine hydrochloride in pyridine (20 mg·mL^{-1}) were added to the dry extract, and the solution was heated at 85 °C for 1 h. After cooling to room temperature, 125 μL hexamethyldisilazane and 5 μL trifluoroacetic acid were added. The sample solution was centrifuged for 5 min at 5900 g_n, then the clear supernatant was withdrawn and transferred to a GC vial.

The analyses were performed with an Agilent Model 6890N GC (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector. One microliter of each sample was injected on a 2.5% phenyl methyl siloxane capillary column (25 m × 0.2 mm i.d., 0.33-μm film thickness; Agilent 19091B-102E). Hydrogen was used as the carrier gas at a flow rate of 0.5 mL·min^{-1}. The injector temperature was held at 250 °C in the split mode (split ratio 50:1). The column oven temperature started at 180 °C for 2 min, increased to 240 °C at 10 °C·min^{-1}, held at 240 °C for 3.5 min, increased to 270 °C at 30 °C·min^{-1}, and held at 270 °C for 12 min. Identification and quantification of sugars were based on retention times and peak areas, respectively, of reference sugars (Sigma-Aldrich Co., St. Louis, MO).

Amino acid analysis. Amino acids were converted to derivatives with *o*-phthaldialdehyde (OPA) and 9-fluorenylmethoxycarbonyl chloride (FMOC) using the method of Herbert et al. (2000), with several minor modifications, before analysis by high-performance liquid chromatography (HPLC). The HPLC system consisted of model HP1090L (Hewlett Packard Co., Rancho Bernardo, CA) fitted with a HypersilAA column (Agilent 79916AA) and a tunable fluorescence detector (HP1046A; Hewlett Packard Co.). For derivatization of primary amino acids, 5 μL of 0.4 N borate buffer (pH 10.2) 1 μL OPA reagent (Agilent p/n 5061–3337), and 1 μL of the extract were introduced into the sample loop and allowed to mix. Secondary amino acids were subsequently derivatized by adding 1 μL FMOC reagent (Agilent p/n 5061–3337) to the system. After derivatization, which required less than 2 min, 1 μL of the sample mixture was injected into the column.

A gradient mobile phase consisted of solvent A and B at an initial flow rate of 0.45 mL·min^{-1}. Solvent A was 20 mM sodium acetate buffer (pH 7.2) with 0.018% (v/v) triethylamine and 0.3% tetrahydrofuran. Solvent B was 20% mM sodium acetate buffer (pH 7.2), 40% acetonitrile, and 40% methanol. The ratio of solvent A to B changed from 100% to 40% within 17 min declining to solvent B alone during the next minute. The flow rate was then ramped to 0.8 mL·min^{-1} in 0.5 min and held at this rate for 4.5 min before returning to 0.45 mL·min^{-1}. Amino acids were detected by a fluorescence spectrophotometer with excitation and emission wavelengths set at 340 and 450 nm, respectively, for the first 15 min and then adjusted to

266 and 305 nm, respectively. An amino acid standard mix (Agilent Technologies) was used for obtaining qualitative and quantitative information.

Other stigma analyses. Stigma exudates of apple flowers at 40% to 60% anther dehiscence were analyzed in 2004 for total carbohydrates, proteins, and lipids. Each sample was derived from 50 flowers as already described, except filtration was performed only with membranes of 0.22-μm pore size and not with devices designed to block macromolecules.

Carbohydrates in five 'Fuji' and five 'Gala' samples were hydrolyzed using the method of Goubet et al. (2002) and then analyzed for monosaccharides. A 50-μL aliquot from each 600-μL sample was heated at 100 °C for 2 h in 0.5 mL of 2 M trifluoroacetic acid, dried in a vacuum evaporator, and put through three methanol wash and dry cycles. It was then subjected to derivatization and GC as described for sugar analysis.

Protein quantities in seven 'Fuji' samples and nine 'Gala' samples were estimated by the method of Bradford (1976) using a kit obtained from Sigma Diagnostics (St. Louis, MO). A 50-μL aliquot per sample was tested. Protein standards from human albumin were prepared at 0.5, 1, 2, and 3 mg·mL^{-1}.

The aqueous stigma samples were evaluated for the presence of lipids by GC analysis of fatty acid methyl esters (Moss, 1981) using procedures specified in the Sherlock Microbial Identification System (MIDI, Newark, DE). This was done with three 'Fuji' and two 'Gala' samples. The 600-μL samples were dried in a vacuum evaporator before esterification. GC equipment was the same as that used for sugar analysis, and GC conditions were set by MIDI software for eukaryotic cells (EUKARY Version 3.71).

Data analyses. Means and SE were calculated for quantities of specific components detected in exudates extracted from the pomaceous stigmas. Comparisons of total free sugars and amino acids from flowers at different stages of anthesis were done by linear regression and *t* tests for two group means using SAS v.9.1 software (SAS Institute, Cary, NC).

Results

During flower sampling from the apple and pear orchard site, daily high and low temperatures averaged 18.7 and 5.5 °C in 2003 and 22.2 and 3.3 °C in 2004. Only a trace amount of rain was recorded on 1 d in 2003 and no rain was detected in 2004. Rate of flower development and senescence was similar in the 2 years. When flowers were newly opened, stigmas appeared light green, and anthers were bright yellow and none dehisced. At 4 or 5 d after petal expansion, when anthers were fully dehisced, stigmas had begun changing from light green to yellow, and petals were beginning to drop. Flowers were generally not collected beyond 5 or 6 d after petal expansion.

At each stage of anther dehiscence, free sugars in stigma exudates from pear, apple, and crab apple flowers were predominately glucose and fructose in near equal proportions with only trace amounts of sucrose (Fig. 1). One exception involved 2004 samples from 'Anjou' pear in which the sucrose portion approached that of monosaccharides (Fig. 1B). Also detected in stigma exudates were low or trace amounts of sorbitol and inositol. Monosaccharides detected in stigma exudates appeared to increase with flower age as indicated by the progression of anther dehiscence (Fig. 2). Average total quantities of monosaccharides extracted from pear stigma exudates ranged from 0.31 μg per flower at early anthesis (0% anther dehiscence) to 1.27 μg per flower at late anthesis (100% dehiscence); monosaccharide quantities for apple at early and late anthesis were 1.87 and 3.19 μg per flower. The data did not fit the linear regression model ($r^2 < 0.25$); however, *t* test comparisons of monosaccharide quantities for early and late anthesis revealed differences at $P < 0.1$ for pear ($P = 0.08$) and apple ($P = 0.06$).

When free sugars in nectar were analyzed in 2004, quantities were a higher magnitude than those indicated for stigma exudates (Fig. 3), as expected, given that nectar secretions are generally present in far greater volumes than are stigma exudates. The amount of sucrose detected in pear nectar was very low relative to monosaccharides; in apple nectar, however, the sucrose level approached that of individual monosaccharides.

Analyses of free amino acids in stigma exudates showed similar results in 2003 (data not presented) and 2004, indicating totals of less than 1 pg extracted per flower. Levels were comparable among cultivars of the same species (data not presented), but values for pear and apple were of a different magnitude. In 2004, total free amino acids from pear flowers at 0%, 40% to 60%, and 100% anther dehiscence averaged 28 (±22), 18 (±1), and 46 (±19) femtograms (fg) per
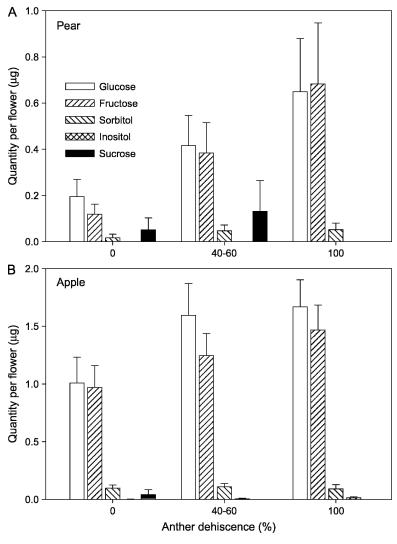

Fig. 2. Free sugars detected in stigma exudates from (**A**) pear (*Pyrus communis*) and (**B**) apple (*Malus pumila*) flowers at three stages of anthesis based on anther dehiscence in 2003 and 2004. Each bar represents mean (±SE) of seven to nine pear samples or 14 to 16 apple samples, each derived from 50 flowers.
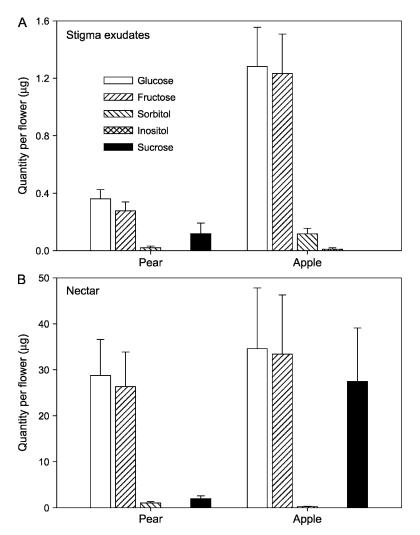
Fig. 3. Comparison of free sugar quantities in (**A**) stigma exudates and (**B**) nectar from pear (*Pyrus communis*) and apple (*Malus pumila*) in 2004. Each bar represents mean (±se) of 12 pear samples or 18 apple samples, each derived from 50 flowers.

flower, respectively; amino acids from apple flowers at these stages averaged 118 (±25), 347 (±5), and 641 (±125) fg per flower, respectively; and those from 'Manchurian' crab apple, represented by single samples at each flower stage, were 20, 99, and 84 fg per flower, respectively. In the case of apple, which involved more replicate samples and higher levels of detectible free amino acids, linear regression analysis data showed that total quantities of amino acids in stigma exudates increased during anthesis (P = 0.0002; r^2 = 0.59). For all flower types, the predominant free amino acids in stigma exudates were proline, asparagine, glutamine, glutamic acid, and serine (Fig. 4); quantities of these specific amino acids extracted per flower at different stages of anthesis are presented in Figure 5. For apple, the increase of predominant amino acids as a group during anthesis was significant according to linear regression ($P < 0.0001$; r^2 = 0.69) and a t test comparison of early and late anthesis (P = 0.0002).

Additional analyses were performed to assess the general presence of carbohydrates, proteins, and lipids in stigma exudates using 'Fuji' and 'Gala' apple flowers at 40% to 60% anther dehiscence. When samples were hydrolyzed and evaluated for sugars, results with both cultivars indicated a predominance of galactose and arabinose (Fig. 6). For 'Fuji', the ratio of galactose to arabinose was 1.43, and for 'Gala', it was 1.54. The only other sugars detectable in the hydrolyzed samples were glucose and fructose; of these, glucose was most prevalent, detectable at levels exceeding those in nonhydrolyzed samples. Total polysaccharides in samples were estimated by subtracting free sugar quantities detected in nonhydrolyzed samples (Table 1). According to these estimates and results of the Bradford tests for protein, quantities of polysaccharides and proteins in exudate samples from 'Fuji' were approximately twice those for 'Gala' (Table 1). Nevertheless, ratios of polysaccharide to protein in extracts from the two cultivars were similar; 1.10 for 'Fuji' and 1.14 for 'Gala'. No lipids were detected in the aqueous extracts.

Discussion

The quantities of chemical components detected in aqueous extracts from the poma-

ceous flowers in this study may not represent total amounts present on stigmas, but reflect differences depending on species and stage of anthesis. When flower stigmas were sonicated for longer than 10 s, levels detected increased (data not presented); it is unknown, however, whether the sonication procedure removes substances primarily from the stigmatic surfaces or forces additional material from secretory cells. Furthermore, some extracted substances may have been associated with pollen, although pollen grains were separated from extraction samples after sonication.

The predominant free sugars detected on three species of pomaceous flowers were clearly glucose and fructose (Fig. 1). Sucrose was detected only in trace amounts, with the exception of 'Anjou' pear in 2004, when sucrose represented 31% of sugar detected in the exudates. Among the three plant species studied, sugar quantities detected were highest for apple followed by crab apple and pear. These differences are likely due in part to differences in stigma size and surface area; stigmas of apple are visibly the largest and those of pear are the smallest.

Before stigma exudates were analyzed for free sugars, we predicted that sucrose would be identified as a major component, since sucrose is generally a significant part of apple nectar (Campbell et al., 1990) and sometimes prevalent in pear nectar (Bieleski and Redgwell, 1980). Nectar sugars from apple were shown to have a sucrose–hexose ratio of 0.4, comparable to ratios reported by Campbell et al. (1990). Only trace amounts of sucrose were detected in pear nectar, however, as consistent with a report by Wykes (1952). Concurrent analysis of free sugars in stigma exudates and nectar from the same flowers validated that sucrose is generally absent in stigma exudates of pomaceous flowers but present as a major component of the nectar, particularly in apple blossoms. Free sugars in nectar had a total mass of between 10× and 100× that in stigma exudates. The hypanthial surface where nectar is present likely is a less favorable habitat for bacteria than the stigma because nectar sugar concentrations vary widely, as dependent on moisture conditions (van der Zwet and Keil, 1979); dry conditions lead to increased sugar concentrations and osmotic pressure, causing a cessation of bacterial growth or decline in viable cell numbers (Ivanoff and Keitt, 1941; Pusey, 1999a, 2000).

The quantities of free amino acids detected were extremely low, relative to sugar levels, yet there was consistency regarding the predominance of certain amino acids for each plant species (Fig. 4). Proline or asparagine was predominant followed by lower amounts of glutamic acid, glutamine, and serine.

As flowers progressed through anthesis, the relative composition of free sugars and free amino acids remained the same but changed quantitatively (Figs. 2 and 5); in general, predominant sugars and amino acids during early anthesis increased and were similarly predominant in late anthesis.
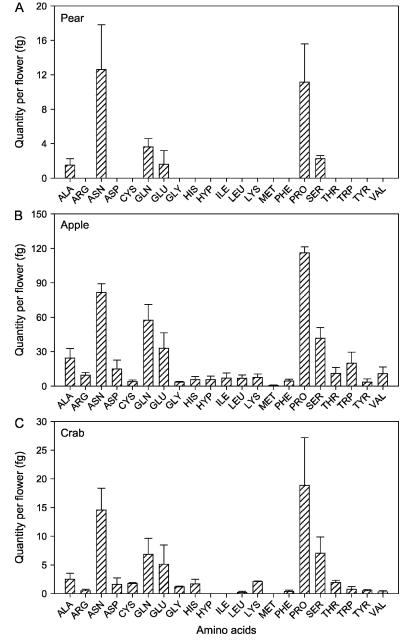
Fig. 4. Free amino acids detected in stigma exudates from (**A**) pear (*Pyrus communis*), (**B**) apple (*Malus pumila*), and (**C**) crab apple (*Malus mandshurica*) in 2004. Each bar represents mean (±SE) of six pear samples, 18 apple samples, or three crab apple samples, each derived from 50 flowers.

large portion of these components, if not all, are in the form of glycoproteins given the frequency of these substances in stigma exudates of other plants (Knox, 1984). Major subcomponents were arabinose and galactose, which are reported often as constituents of glycoproteins in plants, including the arabinogalactan proteins studied extensively as constituents of stigma exudates of *Nicotiana alata* (Cheung and Wu, 1999; Wu et al., 2001). Although levels of free amino acids in apple and pear stigma exudates were very low (Fig. 4), the predominance of proline may be further evidence of glycoproteins, which are often rich in proline or hydroxyproline (Cheung and Wu, 1999). Other predominant free amino acids in the pomaceous exudates are also among those reported to be at highest levels in glycoproteins on stigmas of *Nicotiana* (Gane et al., 1995) and *Brassica* (Nishio and Hinata, 1979). The polysaccharide and protein in extracted stigma exudates from apple may represent variant forms of glycoproteins, including S-RNases (Certal et al., 1999), that possibly interact to perform multiple functions (Cheung and Wu, 1999).

Lipids are often a major component of stigma exudates (Kandasamy and Vivekanandan, 1986; Knox, 1984) and may even be required for directing pollen-tube growth (Wolters-Arts et al., 1998). If lipids are present in apple stigma exudates, as indicated by histochemical studies (Cresti et al., 1980; Heslop-Harrison, 1976; Stant, 1981), perhaps they were not separated from the stigmatic surface by the water extraction method we used (Table 1). It is also possible that quantities of lipids exuded to the surface are relatively low. Stigma exudates of *Gladiolus gandavensis*, which consist of arabinogalactan proteins as the major component (Gleeson and Clarke, 1979), had a detectible lipid content of less than 0.1% (Knox, 1984). In a study with *Aptenia cordifolia* (Kristen et al., 1979), which produces stigma exudates with some of the same carbohydrate and protein characteristics as identified for apple, lipids were not detected. Phenolic compounds are another component of plant stigma exudates (Kandasamy and Vivekanandan, 1986; Knox, 1984), but analyses for these substances were not performed in the present study.

Although free glucose and fructose are minor constituents of the stigma exudates, in terms of mass, these sugars are probably major carbon sources for microbial growth and metabolic activity on pomaceous flowers. On stigmas of individual apple flowers, free monosaccharide quantities available to bacteria are estimated at greater than 3 μg by the time anthers are fully dehisced (Fig. 2). This is sufficient to support maximum bacterial growth on stigmas, known to be between 10^6 and 10^7 cfu per flower (Johnson and Stockwell, 1998) as judged by the relationship of sugar to bacterial yields in enriched laboratory media. The current analyses indicate also that glucose and fructose are increasing on the stigma at the same time that microbial colonies are growing and

Stigmas of rosaceous tree species have been described as having a wet surface, papillate receptive cells, and moderate to slight secretions that flood interstices (Heslop-Harrison, 1976; Knox, 1984). Exudates of apple and pear are present on stigmas from the time flowers first open, apparently originating from cells beneath the papillate epidermis, and subsequently emanating also from the papillae (Heslop-Harrison, 1976; Sanzol et al., 2003). Although histochemical studies indicate the presence of carbohydrates and proteins in rosaceous exudates (Cresti et al., 1980; Stant, 1981), more specific information has been lacking with the exception of recent attention to S-RNases (Certal et al., 1999; McClure and Franklin-

Tong, 2006; Sato et al., 1996; Sonneveld et al., 2005).

Based on the current study, the general chemical characteristics of apple and pear stigma exudates appear to be similar to those of a number of dicotyledonous plants. Secretions of this group are described by Knox (1984) as being "hydrophilic in nature, containing proteins and glycoproteins as major components" and consisting of "free sugars, especially glucose and fructose." Polysaccharides and proteins made up more than 97% of the identified components in aqueous extracts from apple (Table 1), with the polysaccharide-to-protein ratio being ≈1.1, as comparable to *Brassica compestris* (Nishio and Hinata, 1979). It appears likely that a
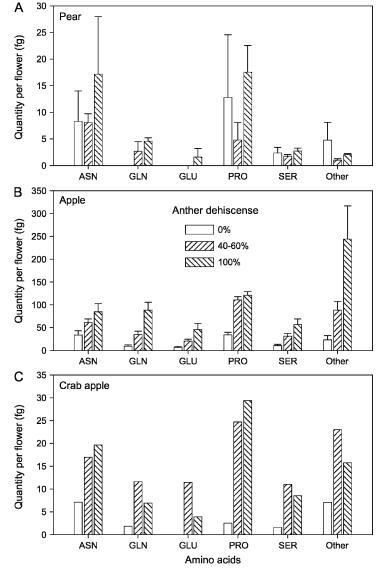
Fig. 5. Free amino acids detected in stigma exudates from (**A**) pear (*Pyrus communis*), (**B**) apple (*Malus pumila*), and (**C**) crab apple (*Malus mandshurica*) flowers at three stages of anthesis based on anther dehiscence in 2004. Each bar represents mean (±SE) of two pear samples, six apple samples, or one crab apple sample, each derived from 50 flowers.
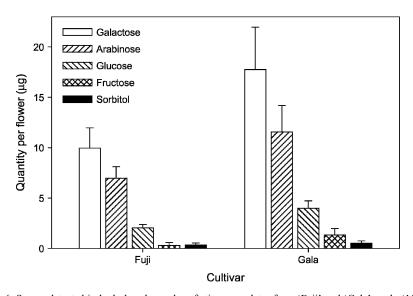

Fig. 6. Sugars detected in hydrolyzed samples of stigma exudates from 'Fuji' and 'Gala' apple (*Malus communis*). Each bar represents mean (±SE) of five samples, each derived from 50 flowers.

increasing their consumption of sugar. The period of anthesis, beginning at petal expansion and typically lasting 3 to 5 d depending on temperature, is about the period when stigmas are most conducive to bacterial colonization and growth (Pusey and Curry, 2004). The levels of free amino acids extracted per flower are not sufficient alone as sources of nitrogen for microbial growth but may be indicative of primary amino acids available from proteins through enzymatic action.

Another example of the applicability of this information to microbial activity on stigmas relates to the interaction between *E. amylovora*, the fire blight bacterium, and *Pantoea agglomerans* [Syn. *Erwinia herbicola* (Löhnis) Dye], another naturally occurring epiphytic bacterium on stigmas. Strains of the latter species have been studied extensively for potential use in biological control (Epton et al., 1994; Johnson and Stockwell, 2000; Vanneste, 1996), and three commercial products consisting of the live bacteria are now available or approved for fire blight in North America (Steinwand and Brohm, 2006; U.S. Environmental Protection Agency, 2006a, 2006b) and New Zealand (Vanneste et al., 2002). In addition to competing with *E. amylovora* for space and nutrients on stigmatic surfaces, these bacteria produce antibiotic compounds that inhibit the disease organism. The antibiotics of *P. agglomerans* are diverse and frequently deactivated by certain amino acids, particularly histidine (Ishimaru et al., 1988; Vanneste et al., 1992; Wodzinski and Paulin, 1994). The present study revealed which amino acids (if any, since all free amino acids were detected at very low levels) may be a factor affecting antibiotic activity and showed that histidine is absent or barely detectable. Conversely, nutrient compounds available to microorganisms can also enhance the production and activity of secondary metabolites. *Pantoea agglomerans* strain E325, selected based on screening bioassays with flowers (Pusey, 1997), produces an inhibitory compound exhibiting high specificity toward *E. amylovora* (P.L. Pusey, unpublished data). Production of the inhibitor in vitro was substantially greater when sugars and amino acids identified as exudate constituents were available than when other nutrient sources were present.

Knowledge of chemical components in stigma exudates of pomaceous flowers opens up the possibility of studying epiphytic organisms on synthetic media that are more comparable chemically to the microenvironment on the stigma. Such investigations could lead to a greater understanding of plant–microbe and microbe–microbe interactions. It may even have application in studies of pollen-tube growth. Sucrose has conventionally been used in pollen viability studies for pomaceous species (Flachowsky et al., 2007; Köpcke et al., 2002; Tiwari and Polito, 1988), but in some cases, use of the monosaccharides, glucose and fructose, may be more appropriate or realistic.

Table 1. Summary of major chemical components of stigma exudates from 'Fuji' and 'Gala' cultivars of apple (*Malus pumila*) obtained by aqueous extraction when flowers were at 40% to 60% anther dehiscence[z].

Category	Major subcomponents	Fuji		Gala	
		Mass extracted per flower (µg)	Percent of total	Mass extracted per flower (µg)	Percent of total
Free sugars	Glucose, fructose	2.9 (0.2)	8.2	1.7 (0.01)	2.7
Free amino acids	ASN, GLN, GLU, PRO, SER	$<10^{-6}$	$<10^{-5}$	$<10^{-6}$	$<10^{-6}$
Polysaccharides[y]	Arabinose, galactose	16.7 (3.8)	48.1	33.4 (8.0)	51.8
Protein	—	15.21 (1.8)	43.7	29.4 (5.0)	45.6
Lipids	—	ND[x]	ND	ND	ND

[z]Values are means (n = 2–9) with SE in parentheses.

[y]Polysaccharide quantities were estimated by subtracting total free sugar from total sugar in hydrolyzed samples.

[x]Lipids were not detected using the gas chromatography analysis procedure of Moss (1981).

The current report involving apple and pear may be the first to provide a chemical characterization of rosaceous stigma exudates that includes the identification of specific free sugars, free amino acids, and polysaccharide subcomponents. It also reveals that certain sugars and amino acids are consistently predominant and increase during anthesis. This basic information not only relates to reproduction within an agriculturally important plant group, but should be useful in understanding the microbial interactions affecting disease development.

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