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Occurrence of Polyphenols, Organic Acids, and Sugars among Diverse Elderberry Genotypes Grown in Three Missouri (USA) Locations

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Abstract

Elderberry (*Sambucus* spp.) is an emerging horticultural crop used in a variety of foods, wines, and dietary supplements. A better understanding of the elderberry juice complex including its putative health-promoting compounds in relation to genetic and environmental parameters is needed. A multi-location planting of nine elderberry genotypes was established in 2008 at three geographically-diverse sites in Missouri, USA. Fruits were harvested from replicated plots 2009-2011, frozen, and later prepared for laboratory analysis. Polyphenols, organic acids, and sugars were quantified by HPLC and the results evaluated for response to genotype, site, and year. The American genotypes ‘Ocoee’ and ‘Ozark’ were consistently higher in chlorogenic acids compared to other genotypes, whereas ‘Ocoee’ was significantly higher in rutin than ‘Ozark’. The European ‘Marge’ was significantly higher in isoquercitrin and other flavonoids compared to most North American genotypes. Significant differences in polyphenols were also detected among sites and production years. Malic, citric, and tartaric acids varied significantly among genotypes, sites, and years, whereas succinic, shikimic, and fumaric acids generally did not. Levels of lactic, acetic, and propionic acids were negligible in most samples. The American genotype ‘Ocoee’ was higher in citric and tartaric acids, while lower in malic acid. The sugars glucose and fructose also responded significantly to genotype, site, and year. ‘Ocoee’, ‘Ozark’, and ‘Marge’ perform very well in Missouri horticulturally and appear to have additional potential as cultivars based on their unique juice characteristics.

Keywords

Sambucus; dietary supplement; fruit; antioxidant; flavonol glycoside

INTRODUCTION

American elderberry [*Sambucus nigra* L. subsp. *canadensis* (L.) Bolli; syn. *S. canadensis* L.] is an emerging horticultural crop in North America that is increasingly used in foods, wines, and dietary supplements. More information is needed on the production of various elderberry metabolites in relation to genetic and environmental parameters, especially as a variety of dietary supplements are being developed and consumed (Charlebois et al., 2010). Because elderberry is predominantly used as a processing fruit, genotypes and cultivars that are consistently higher in certain desirable metabolites across multiple environmental parameters may be preferable for growing and processing. Furthermore, understanding environmental factors that affect elderberry juice characteristics and quality will permit producers and processors to develop more consistent, high-quality food products. Little information on the types and quantities of organic acids and sugars in elderberry is available, especially in the American subspecies. Verberic et al. (2009) quantified carbohydrates and organic acids in European elderberry (*Sambucus nigra* L. subsp. *nigra*; syn. *S. nigra* L.). Sugars detected were mostly glucose and fructose, with minor amounts of sucrose across five genotypes. Organic acids included citric, malic, shikimic, and fumaric. In that study, the popular European cultivar ‘Haschberg’ had lower levels of carbohydrates compared with other genotypes, but was highest in citric and total organic acids. We are not aware of any published studies on levels of these metabolites in American elderberry.

Polyphenols and other anti-oxidants in elderberry have been somewhat more thoroughly studied, including in the American subspecies. Kaack et al. (2008) evaluated chlorogenic acids, flavonol glycosides, and anthocyanins among six European elderberry cultivars harvested at different stages of ripeness. Levels of chlorogenic acids in fruit tended to decrease across the ripening period of late August through mid-September, and they found significant differences in levels among cultivars. The same group (Christensen et al., 2008) quantified similar polyphenols in flowers from 16 European elderberry cultivars and genotypes, again finding significant differences in levels among genotypes. Lee and Finn (2007) evaluated a variety of anthocyanins and polyphenolics in both American and European elderberry that had been grown in Oregon. The European genotypes tended to produce fruit higher in levels of cinnamic acids and flavonol glycosides, with the cultivar ‘Haschberg’ significantly higher in total polyphenolics compared with the other European cultivars and especially the American genotypes. Özgen et al. (2010) studied 14 wild American elderberries brought into cultivation at a single site in Ohio, finding significant differences in levels of total phenolics among genotypes. Thomas et al. (2013) quantified total phenolics in 12 American elderberry genotypes grown in three Missouri environments, also finding significant differences in levels of total phenolics among genotypes, production years, and sites. The present study takes a more detailed look at specific polyphenols found in nine elderberry genotypes grown in a horticultural setting over multiple growing seasons and at multiple sites – a genotype by environment evaluation.

MATERIALS AND METHODS

Field Methods

A planting of nine elderberry genotypes was established in 2008 at three geographically-diverse sites in Missouri, USA: University of Missouri's Southwest Research Center at Mt. Vernon, Missouri State University's State Fruit Experiment Station at Mountain Grove, and Lincoln University's Carver Farm at Jefferson City. One of the genotypes, 'Marge', is of European origin (*S. nigra* subsp. *nigra*) (Thomas et al., 2015b), whereas the eight other genotypes are North American (*S. nigra* subsp. *canadensis*). The plantings at each site were established in a completely randomized design with four replications of four-plant plots per genotype. Details on these plantings, including geography, soils, climate, establishment, plant/row spacing, management, yields, and the elderberry genotypes used are provided in Thomas et al. (2015a,b). Fruits were harvested at peak ripeness from replicated plots in 2009, 2010, and 2011 at Mt. Vernon, and in 2010 and 2011 from Mountain Grove and Jefferson City. Harvested berries were placed into zippered plastic freezer bags and promptly frozen. Later, they were de-stemmed, thawed, and hand-macerated, with juice aliquotted into 5-ml tubes and re-frozen before laboratory analysis. Fruit juice from 2009 and 2010 was stored at −20°C, then analyzed together with fruit juice from 2011 soon after that harvest.

Laboratory Methods

Polyphenols, organic acids, and sugars were quantified by high performance liquid chromatography (HPLC). For organic acid and carbohydrate analysis, elderberry juice samples were prepared by placing 0.5 g homogenized juice into a 5 ml volumetric flask and brought to volume with distilled water. Approximately 1.5 ml of the diluted sample was transferred to a polypropylene micro-centrifuge tube, and centrifuged at 10,000 rpm for 5 min. Approximately 1 ml of supernatant was filtered through a 0.45 μm syringe filter into an autosample vial for HPLC analysis. Organic acids (citric, tartaric, malic, succinic, shikimic, fumaric, lactic, acetic, and propionic) were identified and quantified using an HPLC method adapted from Tessini et al. (2009) with some modifications. The HPLC columns used were an Aminex 87HPX-87H, 300 × 7.8 mm, and an Agilent Eclipse XDB-C18, 5 μm 4.6 × 150 mm (Bio-Rad, Hercules, CA) run in series and maintained at 65°C. The mobile phase was 0.005 M sulfuric acid, flow rate 0.5 ml/min, sample size 20 μl, run time 30 min, with UV detection at 220 nm. The carbohydrates glucose and fructose were quantified using methods adapted from Castellari et al. (2000) with some modifications. The HPLC column was an Aminex 87HPX-87H, 300 × 7.8 mm run at 55°C. The mobile phase was 6% acetonitrile in 0.045 N sulfuric acid, flow rate 0.5 ml/min, sample size 20 μl, run time 30 min, with refractive index detection. Organic acids and sugars were identified by comparing retention times of known standards (Sigma-Aldrich, St. Louis, MO), then quantified by using different concentrations of the standards to develop a standard curve for comparison.

For polyphenols analysis, the procedure of Lee and Finn (2007) was used with slight modifications. Briefly, 0.5 ml elderberry juice was diluted with 3.5 ml 0.1 N HCl, and passed over a Baker SPE C18 column. A gradient of mobile phase A with 2% acetic acid in water and mobile phase B 0.5% acetic acid in acetonitrile:water (1:1) was used. The HPLC column was a Phenomenex Synergi 4 μm Hydro-RP80A, 150 × 2.00 mm with UV detection

at 320 nm. Concentrations were calculated from standard curves for chlorogenic acid and rutin.

Statistical Methods

Elderberry fruit juice data were sampled in the form of a repeated measure over time (three harvest years) from three locations, nine genotypes, and with four field-plot replications per genotype. An analysis of variance was used to evaluate the response of the various metabolites to genotype, site, and year, with means separated by the least significant difference test at $p \leq 0.05$ (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

As described in Thomas et al. (2015a,b), the elderberry plants thrived at all three sites during the study, and notable differences in plant phenology, morphology, yield, and juice characteristics were observed among genotypes, sites, and years. In this more detailed evaluation of fruit from those studies, significant differences were also detected in levels of sugars, organic acids, and polyphenols across the nine genotypes, three sites, and three growing seasons.

Malic, citric, and tartaric acids varied significantly among genotypes, sites, and years, whereas succinic, shikimic and fumaric acids generally did not (Table 1). Levels of lactic, acetic, and propionic acids were negligible in most samples (data not shown). The most abundant organic acids in our samples were malic, citric, and succinic, in that order based on overall means. Verberic et al. (2009) found very similar mean levels of citric acid (3.5 mg/g) in European elderberry compared with the American elderberry in our study (3.15 mg/g), but lower levels of malic acid (1.1 mg/g) compared with our study (3.24 mg/g). Levels of shikimic and fumaric acids were much lower in our study compared with levels found in European elderberry by Verberic et al. (2009). They did not evaluate tartaric or succinic acids. In two other studies of European elderberry, Mikulic-Petkovsek et al. (2012a) and Karovičová et al. (1990) reported mean levels of citric and malic acids at 9.4 and 1.67 g/kg, and 7.34 and 3.04 g/L, respectively. Thus citric acid is considered the predominant organic acid in elderberry based on these studies of European elderberry. The single European genotype in our study, ‘Marge’, was also higher in citric compared with malic acid, and was in the median range among the American elderberry genotypes for malic, citric, and most other acids. However, ‘Marge’ had comparatively very low levels of tartaric acid compared with the American genotypes; low levels of tartaric acid were found in European elderberry by Mikulic-Petkovsek et al. (2012a), but Verberic et al. (2009) did not evaluate tartaric acid, presumably because amounts were considered negligible. In general, levels of organic acids were lower in our Missouri-grown fruit compared with these studies of European elderberry. Our study suggests that American elderberry is somewhat different in organic acid profile compared with European elderberry, but that the Missouri environment may be responsible for an overall reduction in levels of organic acids in ripe fruits. Karovičová et al. (1990) also evaluated organic acids in a different elderberry species, *Sambucus ebulus* L., finding very different levels of citric (1.57) and malic acids (1.05 g/L) compared with *S. nigra* subsp. *nigra* in the same study. Therefore, it may be expected that the genetically-distinct American

elderberry has a somewhat different organic acid profile compared with European elderberry. Among the three sites, Mountain Grove generally produced elderberry fruits with higher organic acid levels across genotypes and years compared with the other two sites. The site and climatic conditions at Mt. Vernon and Mountain Grove are more similar compared with the Jefferson City site which is 150 km latitudinally north of the other sites (in a different USDA Hardiness Zone), and on a more alluvial soil with no fragipan. All sites were irrigated and treated as similarly as possible; therefore it is difficult to explain why berries produced at Mountain Grove were higher in organic acids. Levels of several organic acids also varied among growing seasons, with little discernible pattern emerging.

Levels of fructose and glucose in elderberry fruits were also affected by genotype, site, and year. Sugar levels were highest (statistically equal) in six of the nine genotypes, including the European ‘Marge’, and lowest in ‘Sperandio’, a genotype that has been noted for browning in storage and otherwise poor quality fruit (Thomas et al., 2013). In most cases, the fruits contained somewhat higher levels of fructose (mean 26 mg/g) compared with glucose (22 mg/g), although we did not make statistical comparisons between levels of the different sugars. Two studies of European elderberry fruits grown in Europe (Veberic et al., 2009; Mikulic-Petkovsek et al., 2012a) found somewhat higher levels of fructose (44 and 29 mg/g) and glucose (43 and 26 mg/g), respectively, compared with fruit in our study.

Statistical interactions among experimental variables (genotype, site, and year) were generally insignificant for both sugars and for all organic acids except citric and tartaric. Most notable were significant interactions between genotype and the other factors for tartaric acid, which may have been a result of the very low levels of tartaric acid found in the European ‘Marge’.

Significant differences in polyphenols were also detected among all experimental factors (Table 2). The American elderberry genotypes ‘Ozark’ and ‘Ocoee’ were consistently higher in chlorogenic acids compared with all other genotypes; however, these two genotypes diverged with rutin, where ‘Ocoee’ had significantly higher levels than all other genotypes, and ‘Ozark’ relatively low levels. ‘Sperandio’ and ‘York’ were lowest in the chlorogenic acids, as well as the flavonol glycosides isoquercitrin and rutin. The European ‘Marge’ was generally higher in isoquercitrin. Levels of rutin were the most variable among genotypes, with levels ranging from 38 mg/kg in ‘Sperandio’ to 264 mg/kg in ‘Ocoee’. Levels of the remaining flavonol glycosides were relatively inconsistent within genotypes, except that ‘Sperandio’ tended to have very low levels. Veberic et al. (2009) quantified several polyphenols and anthocyanins in European elderberry, finding levels of these metabolites differing significantly among genotypes. The same group (Schmitzer et al., 2010) evaluated some of the same polyphenols included in our study; in European elderberry must and wine they found levels of 43 and 66 mg/L chlorogenic acid, 15 and 22 mg/L neochlorogenic acid, and 0.7 and 1.1 mg/L kaempferol 3-rutinoside, respectively. These levels were relatively similar to ours for chlorogenic and neo-chlorogenic acids, but much lower than ours for kaempferol 3-rutinoside. Mikulic-Petkovsek et al. (2012b) quantified additional polyphenols in European elderberry that were common to our study, finding mean levels of 2.7, 8.1, and 2.9 mg/kg of isorhamnetin 3-glucoside, isorhamnetin 3-rutinoside, and kaempferol 3-rutinoside, compared with our mean levels of 3.6, 76.5, and 9.2, respectively. Those studies

did not evaluate different elderberry genotypes or growing environments. Kaack et al. (2008), however, compared phenolic acids in six European elderberry cultivars throughout the ripening stage. Chlorogenic acids in berries tended to decline as the ripening period progressed, and neochlorogenic acid in particular showed significant variability due to genotype. Most of the chlorogenic acids and flavonol glycosides in our study were also evaluated by Lee and Finn (2007), who evaluated both American and European elderberry grown at a single site across two seasons in Oregon. In general, we found somewhat higher levels of most cinnamic acids and flavonol glycosides in our Missouri-grown fruit compared with their Oregon-grown fruit. Common to both studies were the American elderberry genotypes ‘Bob Gordon’ (called ‘Gordon B’ in that study; Byers and Thomas, 2011) and ‘York’. In both studies, ‘Bob Gordon’ had somewhat higher levels of most polyphenols compared with ‘York’.

Some significant differences in polyphenols were noted across years, where generally higher levels were found in 2011 juice compared with older juice. While these differences may have been due to varying environmental growing conditions across the three years, they may also have been due to the longer storage time of juice samples from earlier harvests. While anthocyanins and other anti-oxidants are considered unstable under long-term storage (e.g., Kaack, 1989; Kaack and Austed, 1998; Inami et al., 1996), more study is needed to determine how the chlorogenic acids and flavonol glycosides in elderberry juice respond to various storage regimens, including freezing. Responses of polyphenol levels to interactions among experimental factors were inconsistent overall; however interactions involving genotype tended to be significant more often than were interactions involving site or year. The most profound interactions were with isoquercitrin where the factors of genotype, site and year were all highly significant.

‘Ocoee’, ‘Ozark’, and ‘Marge’ perform very well in Missouri horticulturally (Thomas et al., 2015a,b), and appear to have additional potential as elderberry cultivars grown specifically for dietary supplements based on their desirable juice characteristics and consistently high levels of health-promoting metabolites.

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Table 1

Levels of organic acids and sugars found in nine elderberry genotypes from three Missouri sites across three growing seasons (2009-2011).

	Malic acid (mg/g)	Citric acid (mg/g)	Succinic acid (mg/g)	Tartaric acid (mg/g)	Shikimic acid (mg/g)	Fumaric acid (mg/g)	Fructose (mg/g)	Glucose (mg/g)
Year (Y)								
2009	2.57 b [z]	3.09 ab	2.15 b	0.56 c	0.061 a	0.006 a	26.5 a	23.9 a
2010	2.89 b	2.70 b	3.21 a	1.34 b	0.074 a	0.015 a	21.3 b	19.1 b
2011	3.65 a	3.40 a	3.16 a	2.32 a	0.095 a	0.030 a	27.3 a	22.1 a
Site (S)								
MV	3.25 ab	2.94 b	2.74 a	1.44 b	0.111 a	0.022 a	25.0 b	21.6 b
MG	3.76 a	4.06 a	3.35 a	2.68 a	0.042 a	0.022 a	30.5 a	25.1 a
JC	2.92 b	2.97 b	3.21 a	1.74 b	0.059 a	0.022 a	23.2 b	19.4 b
Genotype (G)								
Bob Gordon	2.74 bc	3.01 bc	3.00 a	2.42 ab	0.063 a	0.016 a	29.9 a	23.8 a
Dallas	3.27 b	2.57 c	3.25 a	1.96 bc	0.247 a	0.068 a	23.7 bc	18.2 bc
Marge	2.76 bc	3.61 b	2.40 a	0.61 e	0.099 a	0.008 a	29.8 a	25.8 a
Ocoee	1.94 c	5.01 a	3.61 a	2.73 a	0.088 a	0.005 a	28.0 a	22.4 ab
Ozark	4.41 a	3.11 bc	2.47 a	1.39 d	0.057 a	0.021 a	28.8 a	23.1 a
Ozone	4.25 a	3.17 bc	2.94 a	1.85 cd	0.045 a	0.014 a	28.2 ab	22.4 ab
Sperandio	2.83 b	1.63 d	2.78 a	0.81 e	0.017 a	0.021 a	14.1 d	14.1 c
Wyldewood	4.38 a	2.94 bc	2.91 a	2.51 a	0.074 a	0.022 a	20.0 c	16.5 c
York	2.50 bc	3.24 bc	3.61 a	1.45 cd	0.040 a	0.020 a	26.7 ab	26.0 a
Overall mean	3.24	3.15	2.99	1.77	0.083	0.022	25.5	21.6
Interactions [y]								
Y × S	NS	*	NS	NS	NS	NS	NS	NS
Y × G	NS	*	NS	*	NS	NS	NS	NS
S × G	NS	NS	NS	***	NS	NS	NS	NS
Y × S × G	NS	NS	NS	NS	NS	NS	NS	NS

[z] Means within sub-columns with the same letters are not significantly different according to the least significant difference test ($P \leq 0.05$).

[y] NS, *, ***: Interactions not significant ($P \leq 0.05$), or significant at the $P \leq 0.05$ or 0.001 levels, respectively.

Table 2

Levels of various polyphenols found in nine elderberry genotypes from three Missouri sites across three growing seasons (2009-2011).

	Chlorogenic acid (mg/kg)	Neo-chlorogenic acid (mg/kg)	Crypto-chlorogenic acid (mg/kg)	Isoquercitrin (mg/kg)	Rutin (mg/kg)	Kaempferol 3-rutinoside (mg/kg)	Isorhamnetin 3-rutinoside (mg/kg)	Isorhamnetin 3-glucoside (mg/kg)
Year (Y)								
2009	20.1 b [z]	8.67 b	2.16 b	3.7 c	66.8 b	7.56 b	29.4 c	0.35 b
2010	22.5 b	7.32 b	2.71 ab	10.6 b	88.5 b	6.56 b	49.4 b	1.52 b
2011	49.2 a	15.29 a	4.36 a	31.1 a	190.6 a	11.14 a	107.5 a	5.76 a
Site (S)								
MV	22.2 b	8.31 b	2.77 b	10.7 c	95.7 c	7.60 b	57.2 c	1.58 c
MG	29.4 b	8.96 b	3.17 ab	27.3 b	142.5 b	8.40 b	74.2 b	6.59 a
JC	61.5 a	18.63 a	4.77 a	30.9 a	202.4 a	12.00 a	107.6 a	4.92 b
Genotype (G)								
Bob Gordon	29.0 b	15.52 b	3.35 bc	22.3 bc	121.3 cd	12.17 a	43.9 cd	2.34 cd
Dallas	13.3 cd	6.17 de	2.60 bcd	21.2 bcd	91.6 d	12.64 a	25.7 de	3.69 bc
Marge	24.1 bc	3.24 de	0.94 bcd	27.5 a	217.6 b	10.31 a	146.6 a	3.05 bc
Ocoee	98.2 a	27.95 a	7.48 a	18.9 cde	264.2 a	9.03 ab	69.3 c	2.26 cd
Ozark	92.3 a	28.83 a	9.18 a	25.7 ab	83.6 d	5.24 b	97.6 b	5.93 a
Ozone	23.9 bc	8.65 cd	3.40 b	14.0 e	134.5 c	11.87 a	169.1 a	4.42 ab
Sperandio	4.2 d	0.25 e	0.01 d	6.3 f	37.8 e	4.89 b	22.6 de	0.92 d
Wyldewood	34.5 b	12.72 bc	3.53 b	25.4 ab	199.0 b	5.06 b	14.2 e	4.17 abc
York	4.7 d	1.15 e	0.51 cd	16.2 de	99.2 cd	10.99 a	99.9 b	4.56 ab
Overall mean	36.1	11.74	3.48	20.2	138.4	9.15	76.5	3.55
Interactions [y]								
Y × S	NS	NS	*	***	NS	NS	NS	NS
Y × G	***	**	NS	***	***	**	***	NS
S × G	***	***	NS	***	***	**	***	NS
Y × S × G	NS	**	**	***	*	NS	NS	NS

[z] Means within sub-columns with the same letters are not significantly different according to the least significant difference test ($P \leq 0.05$).

[y] NS, *, **, ***: Interactions not significant ($P \leq 0.05$), or significant at the $P \leq 0.05$. 0.01, or 0.001 levels, respectively.