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Effect of Cultural Management and Plant Age on the Yield, °Brix, and Antioxidant Content of *Aronia mitschurinii* Grown in Maryland

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ABSTRACT: *Aronia mitschurinii* is a fruiting plant that has the potential of becoming the next-generation superfood. The fruit contains high concentrations of flavonoids, polyphenols, and anthocyanins, which are known to be powerful antioxidants. The fruit is regarded for its potential to treat oxidative stress diseases like cancer. Recent studies have proven that this fruit contains significantly more antioxidants than the açai berry and even 40 times more than tomatoes. Here, we report results for developing and optimizing the horticultural management program for growing aronia on Maryland small farms to produce the crop with the highest possible antioxidant capacity, based on observations since 2009. This was achieved by analyzing how plant age, fertilizers, mineral soil amendments, and other factors like disease and pest pressure affect the antioxidant content. This data can help in improving sustainability of local farm businesses by providing them with new alternative and highly profitable crops to grow and process. Analysis and comparison of the fruit yield, soluble sugar content (°Brix), pH, total polyphenols, total anthocyanins, and total flavonoids of aronia based on two treatment levels of nitrogen fertilizer (3 g N plant^{-1} year^{-1} vs 14 g plant^{-1} year^{-1}) and conventional and organic-based nitrogen are presented. Plants were fertilized with either 127 g (rows A and C) or 27 g (rows B and D) of Bartlett's Boost Natural at the base of each plant. This equated to 14 and 3 g of N, respectively. Average yields of plants given 14 g of organic N were only significantly higher than those given only 3 g of conventional N but not organic N. The yield in all plants increased year by year from 2009 and until 2019 and slightly decreased in 2020. Even though an increase in the anthocyanin content was noted for a lower N rate, the higher N rate would have produced more fruit and hence more anthocyanin per hectare. A higher nitrogen (N) rate positively affected the yield, but not always the phytochemical content. Organic N did not have a positive effect on the phytochemical content. Additionally, we report the cyanide content of aronia fruit in comparison to other fruits.

Sample	Natural pH	APC, mg C3GE/g juice	Flavonoids, mg QE/g juice	Polyphenols, mg GAE/g juice
Ripened Aronia	3.47	2009.745	90.384	595.047
Unripe Aronia	3.37	940.628	29.919	425.711
Peach	4.16	0.384	7.675	155.729
Blueberry	3.13	14.236	10.292	155.585
Blackberry	3.70	28.791	14.491	150.145

INTRODUCTION

Aronia mitschurinii (Figure 1) is a fruit-bearing shrub resulting in the hybridization of *Aronia melanocarpa* (commonly known as black chokeberry) with European mountain ash (*Sorbus aucuparia*) by Ivan Mitschurin in the 19th century.[1] *A. mitschurinii*, referred to as aronia hereinafter, is a member of the Rosaceae or rose family, and it grows 1.8−2.4 m in height.[2] It is an ideal plant for organic production because of its resistance to pests and diseases. Aronia contains a deep-purple pigmentation that arises from its dense content of phenolic phytochemicals, especially anthocyanin, which is a potent antioxidant.[1] It is proven that aronia contains more than 5 times more flavonoids than cranberry and over 40 times the antioxidant trapping ability than tomatoes.[3] These phenolic compounds highlight its antioxidant potential, which has stirred

Figure 1. *A. mitschurinii*.

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the interest of scientists to study its ability of capturing free radicals in the body. This fruit is used to make juice either alone or blended with other fruit juice and is used for making jams, wine, syrup, tea, food coloring, and food supplements.[4]

The fruit has high concentrations of flavonoids including anthocyanins and proanthocyanidins.[5] Anthocyanin is a type of flavonoid glycoside that is widely used as a water-soluble plant pigment in food products.[6] Anthocyanin pigment has been associated with the bright-purple, red, and blue colors of flowers, leaves, and fruits.[7] Anthocyanin pigments are important to food quality because of their contribution to color and appearance in addition to the health benefits of antioxidants. Anthocyanin pigment content can also be a useful criterion in quality control and purchase specifications of fruit juices, nutraceuticals, and natural colorants.[8] Anthocyanins are the glycosidic forms of anthocyanidins.[9] Flavonoids are a group of polyphenols that may be able to prevent cancer and inhibit low-density lipoprotein oxidation induced by free radicals.[10] Phenolic compounds are plant metabolites found in fruits and vegetables; they contribute to the plant's nutritional content. Polyphenols are the main class that contains all of the flavonoids, and anthocyanin gives the plant the ability to capture free radicals.[11] The juice of wild *A. melanocarpa* is known to contain high concentrations of polyphenols up to 7 g/L.[12] Research has also shown that these polyphenols have antiviral agents and can be used as dietary supplements.[13] Due to health-promoting effects, there is great interest in fruits and vegetables containing high concentrations of flavonoids.[5] High-performance liquid chromatography (HPLC) chromatograms have revealed that aronia contains several flavonoids.[14] Wild aronia has a total oxygen radical absorbance capacity (T-ORAC) of 16,062 μmoles Trolox equivalents (TE) per 100 g of fresh fruit. In comparison, commercial blueberries have only 4,669 μmoles TE per 100 g of fresh fruit.[15]

Due to the size of aronia fruit (approximately 1 cm in diameter) and its ability to maintain its nutritional content under very low temperatures, it is possible to freeze-dry the fruit for long-term preservation. This makes aronia an ideal lightweight food supplement for the National Air and Space Administration (NASA) astronauts or for the United States Department of Defense MREs (meals ready to eat). However, aronia also contains small amounts of sorbitol, which, if consumed in large doses, may act as a laxative.

Several research institutions and government agencies have promoted the production of aronia as an alternative crop.[16] In Eastern Europe, aronia products include juices, extracts, coloring agents, and wine.[17] According to data from the Mid-West Aronia Association and the Mid-Atlantic Aronia Growers Association, over 200 farms are growing aronia for profit in the United States. Many of these farms have turned to organic certification to increase the value of their fruit, but little is known about how much organic fertilizer to use to maintain a profitable yield.

Research has shown that the nature of crop treatment, type of soil, age of plant, and the plant cultivar can influence the antioxidant capacity of the fruits.[18] Certified organic fertilizers are more costly than conventional synthetic fertilizers, and rate studies are important for determining optimal application amounts for profitable yields. Jeppson[9] showed that different rates of nitrogen (N) applied to aronia plants influenced the yield and quality of the fruit. Although higher N rates improved yields, the fruit quality suffered. Skupień and Oszmański also showed that the phytochemical content in aronia fruit can be manipulated by fertility.[19] These practices listed above are what are referred to as cultural management techniques. In this case, these are traditional cultural management techniques.

During the 2020 Covid-19 pandemic, novel ways to test and produce effective viricidal products were undertaken. Research into natural consumables that can reduce viral loads in oral and oral-pharyngeal surfaces was conducted by the Cold Spring Harbor Laboratory using a variety of natural products including green tea, and the juices of elderberry, pomegranate, and *A. melanocarpa* were studied. After a 2 or 5 min swirl of either the green tea or fruit juice in the mouths of test subjects, it was determined that aronia juice was the most effective antiviral of those tested by inactivating IAV (swine flu virus) by 99.99%, SARS-CoV-2 by 96.98%, and the control virus by 93.23%.[20]

Here, we report nine years of cultural management studies, including nitrogen rate, nitrogen quality, and mineral amendments on fruit yield, °Brix, antioxidant content, and other phytochemical productions in the fruit.

MATERIALS AND METHODS

General Procedures. Chemical reagents used included aluminum chloride (99% extrapure, anhydrous, granules), ethyl alcohol (99% ACS spectroscopic grade), and quercetin hydrate (95%), which were purchased from Thermo Fisher Scientific. Potassium acetate (certified ACS crystalline) and sodium carbonate anhydrous (HPLC-grade powder) were purchased from Fisher Scientific. Folin and Ciocalteu's phenol reagent and gallic acid monohydrate (ACS reagent grade) were purchased from MP Biomedicals (Santa Ana, CA). Sodium acetate (Sigma Ultra minimum 99.0%) was purchased from Sigma-Aldrich. Conc. HCl, conc. NaOH, pure ethanol, and 95% ethanol were purchased from Fisher Scientific. Distilled water was used for all procedures. Ultraviolet/visible (UV/vis) determination of total concentrations of anthocyanins, flavonoids, and polyphenols was performed on a Spectronic 10 Genesis spectrophotometer, as described in the procedures below.

Fruit Production Location and Plant Fertility Treatments. Aronia samples for this study were obtained from the University of Maryland's Wye Research and Education Center (WyeREC) in Queenstown, MD. The soil was a Mattapex−Butlertown silt loam with 0−2% slope and a pH of 6.1. The soil was maintained sod for longer than a 10-year period before planting. Soil tests revealed adequate amounts of all nutrients except for potassium, which was applied with nitrogen. Soil tests showed the above optimal phosphorus levels (78 ppm from Mehlich III extraction).

Plot B Aronia Planting. In June 2010, 80 aronia "Viking" plants, sourced as 12 month-old rooted cuttings, were planted at a spacing of one meter between plants through a geotextile weed barrier in four parallel rows (A−D) measuring 23 m in length and 1 m wide and spaced 3 meters apart. The experiment was set as a randomized complete block design (RCBD) with rows A and B in block one and rows C and D in block 2. The blocking was developed to try and explain the growth variation noted on previous plots, possibly due to irrigation variability or soil compaction. Plants were initially fertilized at planting with 64 g plant^{-1} of Bartlett's Boost Natural 11-0-4.15 (F.A. Bartlett Tree Expert Company, Stamford, CT), an Organic Material Review Institute (OMRI)-certified organic fertilizer. This equated to 7 g of nitrogen. After the first season's establishment period, the plants were fertilized with either 127 g (rows A and C) or 27 g (rows B and D) of Bartlett's Boost Natural at the base of each plant. This equated to 14 and 3 g of N, respectively. The same N

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ACS Omega 2023, 8, 4060−4071

fertility rates were continued each spring before bud-break (early March) each year during the study between 2010 and 2021. In March 2013, AZOMITE (AZ), an OMRI-certified mineral soil amendment, was added to the fertilizer regimen for 3 years and discontinued in 2017.

Plot C Aronia Planting. In June 2011, 44 aronia "Viking" plants, sourced as 12 month-old rooted cuttings, were planted at 11 plants per row with a spacing of 2.1 m between plants (half the density as Plot B) through a geotextile weed barrier in four parallel rows measuring 23 m in length, 1 m in width, and spaced 3 m apart. The experimental design for Plot C was also an RCBD for the same reasons as Plot B. The intent of Plot C was to quantify differences in fruit yield and quality as related greater canopy-sunlight interception from a lower density planting in combination with N fertility. After the initial fertilization of 7 g of N per plant at planting, plants were not fertilized until March 2013 when N fertility was split into 14 g of N per plant in rows A and C and 3 g of N per plant in rows B and D. An additional treatment, conventional (synthetic) versus organic, was nested in the N rate treatments. Half the plants in each row received the conventional fertilizer, Scott's Topdress with IBDU and sulfur-coated urea with 50% water-insoluble nitrogen (22-2.2-4.9) (Scotts Company, Marysville, OH), and the other plants in each row received the organic fertilizer Bartlett's Boost Natural (The F.A. Bartlett Tree Expert Company, Stamford, CT). Since the organic fertilizer had more potassium (K) content, potassium sulfate (The Andersons, Inc., Maumee, OH), with an NPK ratio of 0-0-41.5, was applied at a rate of 7.4 g of K per plant in row C and 1.6 g of K per plant in row D. The conventional fertilizer contained more phosphorus (P). An organic P source, Earth safe Organics, an OMRI-certified rock phosphate (Carl Pool Products, Gladewater, TX) with an NPK ratio of 0-1.3-0, was used to balance the P for the organically fertilized plants at 1.4 g of P in row C and 0.3 g of P in row B. As in Plot B, fertilizers were placed at the base of plants.

Harvest Sampling and Sample Storage. Starting in 2013, fruit was harvested from plants in mid-August from both Plots B and C. Fruit from each plant was picked by hand, and the total fruit yield from each plant was measured on an Ohaus Explorer Pro CP3200C1 balance (Parsippany, New Jersey).

Sample Preparation. After harvest, the fruit was kept in a freezer at −25 °C before juicing. The samples were then left at room temperature for approximately 4 min to partially defrost. The samples were then weighed to obtain the initial mass to determine the percent yield of juice from the sample. The samples were then juiced by placing the berries in a mortar and ground with a pestle. The paste was then transferred to a gravitational filtration apparatus for vacuum filtration that lasted for about 15 min with occasional pressing of the sample. The juice was collected and weighed on an analytical balance. After all juicing had ceased, juice was placed into several 1.5 mL Eppendorf vials and frozen until the analysis for antioxidant content was performed in triplicates, as described below.

Determination of Cyanide Content. A sample of aronia fruit from Plot B with stems and the peduncle was collected a month before harvest in 2013. Approximately 2.84 g of stem and peduncle with the fruit removed was chopped finely, soaked in 100 mL of ethanol, and allowed to run in a reflux apparatus at 70 °C for 5 h. After the initial reflux operation, the stem material was soaked for 19 h before adding another 30 mL of ethanol and repeating the reflux a second time. The solution was filtered through an 11 μm filter. The solvent was evaporated on a Büchi Collegiate Rotavapor and Heating Bath rotor vacuum evaporator. The pressure of the system and the temperature of the water bath were adjusted to induce reflux and boiling of the solvent at a low temperature. The sample was concentrated to 8 mL. This sample was utilized for cyanide content analysis.

Measurement of pH. The pH of the juice was measured on a calibrated Mettler Toledo FE-20 FiveEasy Benchtop pH meter according to the manufacturer's instructions.

Percent Yield of Juice. Percent yield of juice was calculated after juicing. This used the mass of juice collected in parallel to the mass of the initial fruit sample. The juice trapped within the filter paper was neglected to account for the small amounts of juice lost during production at larger facilities. No significant changes were observed in the yield of juice with regard to treatment.

Measuring the Total Anthocyanin Content. Measurement and calculation of anthocyanin pigment concentration were performed based on the procedure outlined by Lee et al.[21] After defrosting in room-temperature water, samples were vortexed. A portion of each sample was diluted 2000 times into 0.025 M aqueous KCl and onto 0.4 M aqueous sodium acetate. The UV/vis absorbance of each dilution was read at 520 and 700 nm. Performance of the instrument was validated by comparison measurements using a PerkinElmer Victor 3 1420 spectrophotometer.[22] The anthocyanin pigment concentration (APC) was calculated as cyanidin-3-glucoside equivalents in mg/L using the equation

$$\mathrm{APC} = \frac{A \times M_W \times \mathrm{DF} \times 10^3}{\varepsilon \times 1}$$

where A = absorbance = (A520 − A700 nm) pH 1.0 − (A520 − A700 nm) pH 4.5; molecular weight (M_W) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor (200×); 1 = pathlength in cm; ε = 26,900 L/mol/cm, the molar extinction coefficient for cyd-3-glu; and 10^3 = factor for conversion from g to mg.

Measuring Total Flavonoids. The flavonoid content was measured using a method based on those published by Wiosky and Salatino[23] as well as Chang et al.[24] Standards of 0, 30, 60, 90, 120, and 150 μg/mL were made from a dilution of 0.005 M quercetin stock solution in 95% ethanol. Samples were prepared for measurement by the creation of a solution with the following ratios by volume: 1% sample, 39% acidified ethanol, 2% $AlCl_3$, 2% potassium acetate, and 56% distilled water. Components were added and mixed, one by one, in order. The solution was incubated for 5 min at room temperature following both the addition of $AlCl_3$ and the addition of potassium acetate. The final solution was incubated as before for 20 min. Absorbance of both standards and samples was measured at 405 nm using a PerkinElmer Victor 3 1420 Multilabel Counter. Readings of standards were used to make a calibration curve for the calculation of sample values. Flavonoid concentration was expressed as μg quercetin equivalents/mL. This concentration was converted to mg quercetin equivalents based on sample volume.

Total of Polyphenol Measurement. The total polyphenol content was measured using a method published by Kim et al. (2009).[25] Standards of 0, 30, 60, 90, 120, and 150 μg/mL were prepared from a gallic acid stock solution in 80% ethanol/20% deionized water (DI). Experimental samples were prepared for measurement by mixing 198 μL of distilled water with 2 μL of aronia juice and adding 200 μL of each of the gallic acid standards, in turn. Analysis was conducted in triplicate. To each

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ACS Omega 2023, 8, 4060−4071

sample, 1250 μL of Folin's reagent was added. Samples sat for 5 min at room temperature. Then, 1500 μL of 7%(w/v) $NaCO_{3(aq)}$ was added, and the resulting solutions were incubated at 40 °C in an oven for 15 min. Before measuring the absorbance, all samples were cooled in a refrigerator for 5 min. A spectrophotometer was used to measure absorbance at 750 nm. The standards were used to make a calibration curve for the determination of experimental concentrations. All polyphenol concentrations are expressed as μg gallic acid equivalents/mL of juice.

°Brix Measurements. Fruit soluble sugar content (°Brix) was measured from 2013 to 2017 with an autotemperature compensated refractometer (ATAGO Model 2311, ATAGO Co, Tokyo, Japan) after harvest by taking individual juice samples of 10 fruits from the harvest subsample from each plant. In 2018, °Brix was measured with an autotemperature compensated refractometer (Fisherbrand HDR-P1, Thermo Fisher Scientific, Bridgewater Township, NJ) after harvest by taking individual juice samples from 10 fruits from the harvest subsample.

Determination of Cyanide Content in Juice. The method employed was based on spectrophotometric determination modified from Epstein (1947).[26] A 0.001 M KCN stock solution was diluted with water to give final KCN concentrations of 0−100 μg/mL. 27% cyanoline blue (1-phenyl-3-methyl-5-pyrazolone and 4,4′-bis(1-phenyl-3-methyl-5-pyrazolone) at 12:5) in pyridine/water (5:1) was used as the indicator dye for detecting the CN^- in solution. This dye was prepared weekly and stored at 4 °C after incubating at room temperature overnight on day one. A 100 μL 1% Chloramine-T solution was added to 500 μL of standard or undiluted sample in a glass vial with a stopper. The vial was stoppered immediately after addition due to the HCN gas that would be produced. Each vial received 3 mL of 27% cyanoline blue dye, and the standards were incubated at room temperature for approximately 17 min. The samples initially required centrifugation on a Fisher Scientific Centrific Centrifuge for 10 min at a maximum speed and were incubated at room temperature for approximately 7 min before spectrophotometric measurements. Absorbance was read on a UV/vis spectrophotometer at 630 nm. Results were reported as μmol HCN/g juice.[26]

LCMS Analysis of Anthocyanins. Liquid chromatography-mass spectrometry (LCMS) analysis was conducted in the service laboratory of the University of Maryland Baltimore County. Acid hydrolysis was performed on samples of aronia juice prior to analysis to break the glucoside bonds. A total of 1000 μL of methanol with 3 M HCl was added to 500 μL of each juice sample. The sample solution was then placed in boiling water for 20 min. After that, samples were centrifuged for 5 min. The supernatant was filtered using a 0.45 μm nylon syringe filter. Then, 100 μL of the filtered sample was added to 100 uL of methanol and placed into a glass insert for LCMS analysis. PDA detector wavelength acquisitions at 240, 280, 415, and 520 in accordance with appropriate wavelengths for analytes

LCMS parameters were as follows.

- Column: Phenomenex, Gemini NX C18, 5 μm, 110 A, 250 mm × 4.6 mm, Serial No. 725758-5
- Column Temperature: 25 °C
- Flow Rate: 1.0 mL/min
- Injection Volume: 3 μL

Table 1. Gradient Method (A) and MS Method (B) for Determination of Anthocyanins in *A. mitschurinii* Juice

A:		
time (min)	%A	%B
0.0−15.0	70	30
15.0−20.0	60	40
20.0−30.0	35	65
30.0−50.0	20	80
50.0−52.0	0	100
52.0−60.0	0	100
B:		
spectra per sec.		1
acq. function		pulse
polarity		positive
m/z range		150−800
ion source		ESI
cylinder (V)		−3500
endplate (V)		−3800
capillary entrance (V)		−5200
drying gas flow (L/min)		15
drying gas heater (°C)		310
nebulizer gas (psi)		80

- The gradient method was used with mobile phase A as water/acetonitrile = 19:1 and mobile phase B as methanol, as shown in Table 1.

Statistical Analysis. Data including yield, °Brix, and phytochemical content from fruit assays was analyzed according to the experimental procedures described earlier under harvest methods. Inference from all results relied on statistical analyses performed with ANOVA by SAS Proc Mixed (SAS Institute, Cary, N.C.). The block effect was determined to be significant in explaining variation. If treatment interaction was not significant, main effects were reported and discussed. However, if treatment interactions were significant, simple effects (the effect of a variable at a specific level of another variable) were reported and discussed. Data showing unequal variation was reanalyzed.

RESULTS AND DISCUSSION

Comparison of Aronia to Other Fruits with High Antioxidant Content. It has been reported for *A. melanocarpa* that the antioxidant content of this fruit is significantly higher than that of other fruits known for being a good source of antioxidants.[27] To provide more accurate comparisons with the cultivated aronia (*A. mitschurinii*, as compared to *A. melanocarpa*), we analyzed samples of this fruit in the lab and compared it to several other fruits that are commonly considered good sources of antioxidants. Results are presented in Table 2.

As can be seen in Table 2, juice from unripe aronia fruit has a much higher content of all hydrophilic antioxidants compared to other fully ripened fruits. The difference is the most remarkable for anthocyanins, a very valuable phenolic antioxidant.

Percent Yield of Juice. No significant changes were observed in the yield of juice with regard to treatment.

Influence of N Treatment on Fruit Yield. Fertilizer rate had a significant effect on yield after the third growing season after planting for both Plots B and C. Jeppsson[9] suggested that a medium N rate of 50 kg ha^{-1} resulted in optimized anthocyanin

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Table 2. Comparison of the Antioxidant Contents of Aronia Juice with Juices of Other Fruits

sample	natural pH	APC, mg C3GE/g juice	flavonoids, mg QE/g juice	polyphenols, mg GAE/g juice
ripened *A. mitschurinii*	3.47	200.975	90.384	595.047
unripe *A. mitschurinii*	3.37	94.0628	29.919	425.711
peach	4.16	0.384	7.675	155.729
blueberry	3.13	14.236	10.292	155.585
blackberry	3.70	28.791	14.491	150.145

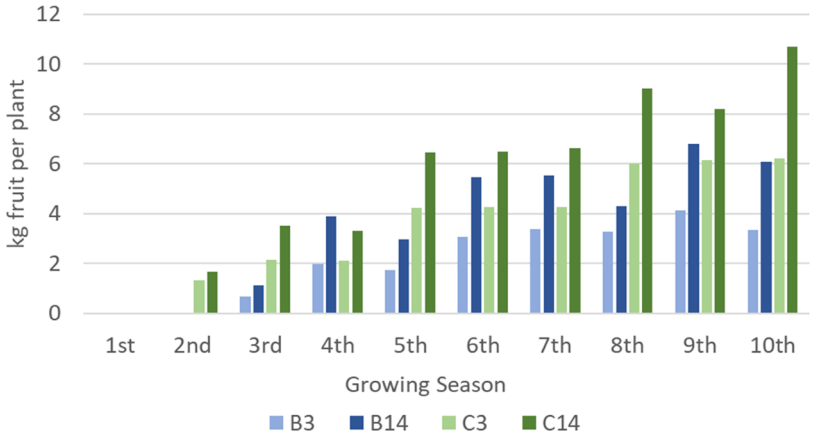

Figure 2. Average kilograms of fruit yield per plant over nine growing seasons. Data from Plot C is aggregated from N rate and N quality. Plots B and C were planted 1 year apart, and the figure normalizes the yields by growing season to compare yield differences from both N fertility and plant spacing. In the first growing season, no appreciable harvest was measured for either plot. In the second growing season of Plot B, there was no yield due to canopy loss from Japanese beetle predation. B and C indicate Plot, and 3 and 14 indicate N rate in grams per plant.

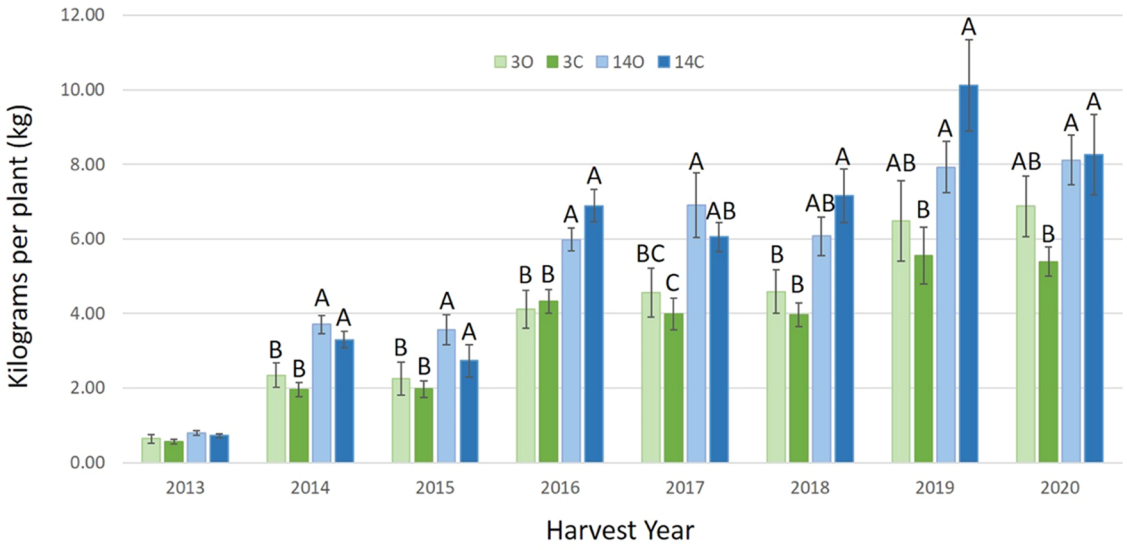

Figure 3. Average annual fruit yield per plant for each treatment in Plot C from 2013 to 2020. Nitrogen treatments per plant are as follows: 3O is 3 g of N in organic form, 3C is 3 g of N in conventional form, 14O is 14 g of N in organic form, and 14C is 14 g of N in conventional form. Different letters within harvest year indicate significant differences between treatments ($p \leq 0.5$). Bars without letters indicate no differences in that year. Error bars indicate one standard error.

content and yield. However, Jeppsson manually applied N, possibly banding (application of a line of fertilizer down the plant row). In this study, fertilizer was applied directly to the base of the plant. Normalizing between studies for comparison, our N treatments would have been approximately 7.8 and 36.4 kg ha^{-1} based on N rates of 3 and 14 g plant^{-1}, respectively, at a plant density of 2600 plants ha^{-1}. These application rates may seem much lower than those of Jeppsson's. However, in our study, N was applied directly to the plant, localizing and increasing the concentration of the fertilizer. Results here show that N fertilization of 14 g plant^{-1} increases the yield more significantly than 3 g plant^{-1}. It is possible in the year immediately following establishment that a N rate of 7 g plant^{-1} is adequate until the third growing season, at which time increasing the N rate to 14 g plant^{-1} (along with other nutrients based on soil tests) would improve the yield.

Jeppsson[9] began data collection 3 years after planting, and yields at high N rates were lower in comparison with this study. This suggests that the aronia plants in this study were not necessarily underfertilized, regardless of the N rate. Only one

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refereed publication documented aronia yields of over 24 kg of fresh fruit per plant 4 years after establishment.[28] This study documented averages of less than 3 kg fruit per plant 3 years after establishment and less than 5 kg plant^{-1} 4 years after establishment. It is possible that geography and climate may also play a role in plant growth and yield based on the comparison of yield results between our research and that of Strik,[32] along with differences in N fertility.

It can be concluded that age of a plant does significantly influence the overall yield of fruit produced by a plant, and N treatment differences were noted after the 3rd growing season. However, yields leveled and remained steady with small fluctuations by the 5th growing season. Results show that application of 14 g N plant^{-1} produces higher yields than plants given 3 g of N. The treatments in this study were applied on a per-plant basis, but the rates could be extrapolated to unit area by estimating the density of plants per unit area. In Plot C, plant density was approximately 1,400 plants ha^{-1}. This equated to approximately 4.2 and 19.6 kg ha^{-1} for 3 and 14 g of N, respectively.

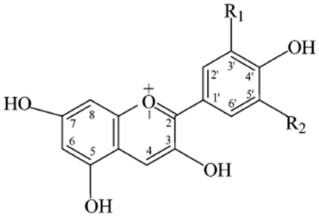
Results presented in Figure 2 represent a comparison of average fruit yield between 2013 and 2020, in average kilograms per plant given either 3 g or 14 g N plant^{-1} and between dense and spaced plantings (Plot B vs Plot C, respectively). The N forms (organic or conventional) are aggregated into N rate since the N form did not have a significant effect on yield. Figure 2 also normalizes the harvest comparison between plots by representing the growing season since Plot B is one year older than Plot C. The first harvest for these two plots is in the year 2013. That would have been the third year after planting for Plot B and the second year after planting for Plot C. In 2012, the orchard was defoliated by Japanese Beetle and no harvest was recorded for Plot B, so there is no 2nd year harvest for plants in Plot B. Also, yields for Plot C are not differentiated between organic and conventional nitrogen, but rather the treatment combination is aggregated between N rates so yield between plots can be compared more easily.

Figure 3 represents a comparison between N rate (3 and 14 g N plant^{-1}) and shows the average yield per plant between 2013 and 2020, between N rates, and between N forms in Plot C. Differences in average plant yield appeared between 3 and 14 g N plant^{-1} in 2014 and continued throughout the study. There were no differences in average plant yield between conventional and organic N in each year regardless of the N rate. Soil potassium (K) was low and therefore was applied with N from the fertilizer with the higher N rate having a higher K. However, leaf analyses did not show plant-K deficiencies between 3 and 14 g N treatments. The differences in K rate between treatments would not have confounded the yield results. In 2013, the plants yielded between 0.1 and 0.4 kg of fruit per plant. Between 2014 and 2015, plant yield increased significantly from 2013 but was not significantly different within the same rate and form among years. For instance, plants given 14 g of organic N did not differ in yield from 2014 and 2015. Another significant increase in yield occurred in 2016, with the yield not changing significantly through 2018, and again, there was no significant difference within the same rate and form within these years. In 2019, another significant increase in yield occurred, especially with 14 g of conventional N, which was significantly higher than the other three treatments. The average yield of plants given 14 g of organic N was only significantly higher than plants given 3 g of conventional N but not organic N. In 2020, the average yield decreased only with plants given 14 g of conventional N.

Plants in Plot B have a lower yield than plants in Plot C within the same N treatment (except in the 4th harvest year). This is due to Plot B's high-density plantings and lower sunlight interception, reducing the yield per plant. However, because Plot B has more plants per hectare than Plot C (approximately 2600 plants vs 1400 plants, respectively), yield per acre is greater in Plot B most years by N rate. If Plot B plants yield 54% or more than the plants from Plot C, the higher density planting produces more fruit. More importantly, this has profound implications regarding anthocyanin production per hectare. Higher plant yield equates to higher phytochemical production per hectare, as discussed in the following section.

Influence of N Treatment on Fruit Quality. Just as the N rate may influence harvested fruit yields, they can also influence a fruit's phytochemical content, which we refer to as quality. The effects of the following treatments: 3 g of organic nitrogen fertilizer, 3 g of conventional nitrogen fertilizer, 14 g of organic nitrogen fertilizer, and lastly 14 g of conventional nitrogen, had some impact on aronia fruit quality.

LCMS Analysis of Anthocyanins in *A. mitschurinii* Juice. Anthocyanins are very potent antioxidants with the general formula presented in Figure 4. Depending on the substitution, there are six most abundant anthocyanins reported in fruits: Pelargonidin, Cyanidin, Peonidin, Delphinidin, Petunidin, and Malvidin.



Anthocyanidin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Peonidin	OCH_3	H
Delphinidin	OH	OH
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 4. Structure of anthocyanins.

Juice of *A. mitschurinii* was hydrolyzed as described above to separate anthocyanins from glycosides. Then, diluted samples and standards for four major anthocyanins were analyzed using LCMS instrument with a triple quad MS detector. The standards were cyanidin, delphinidin, peonidin, and malvidin. The method and results for other fruits are widely described in the literature. Blueberry extracts contain almost even amounts of cyanidin, delphinidin, and petunidin and trace amounts of peonidin and malvidin.[29] Blueberries contain a significant amount of all six anthocyanins, while blackcurrants only contain cyanidin and delphinidin, and strawberries contain cyanidin and pelargonidin.[30] The same study has shown that blackberries, red currants, raspberries, and sour cherries contain cyanidin.

Our results have indicated that *A. mitschurinii* juice, likewise blackberries, raspberries, and sour cherries, contains cyanidin only, as indicated on the chromatogram in Figure 5. Cyanidin's identity was also confirmed with the 520 nm PDA spectrum. Based on this result, further determination of anthocyanin content in aronia using UV/vis methods for total anthocyanins would be as accurate as HPLC/LCMS determination.

Effect of Cultural Management on Phytochemical Content. We observed a significant effect from the amount of nitrogen and the form of fertilizer on certain phytochemicals, by treatment and by harvest year.

Measurement of pH. The pH of the juice for all treatments in both plots ranged between 3.7 and 3.8. There were no treatment effects, and the results are not graphically shown.

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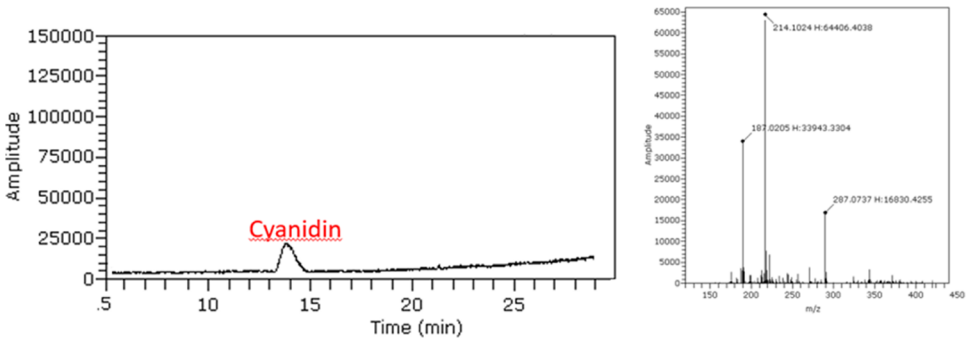

Figure 5. LCMS chromatogram for aronia juice.

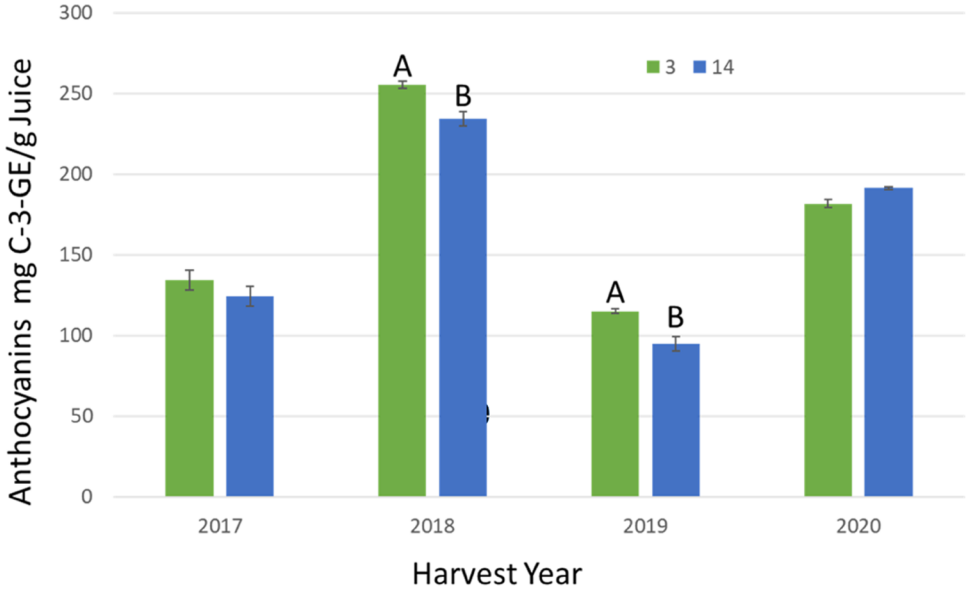

Figure 6. Average anthocyanin content of fruit juice from plants between treatments in Plot B from 2017 to 2020. Plants were treated annually with either 3 g of N (green) or 14 g of N (blue). Different letters within harvest year indicate significant differences between treatments ($p \leq 0.5$). Bars without letters indicate no differences in that year. Error bars indicate one standard error.

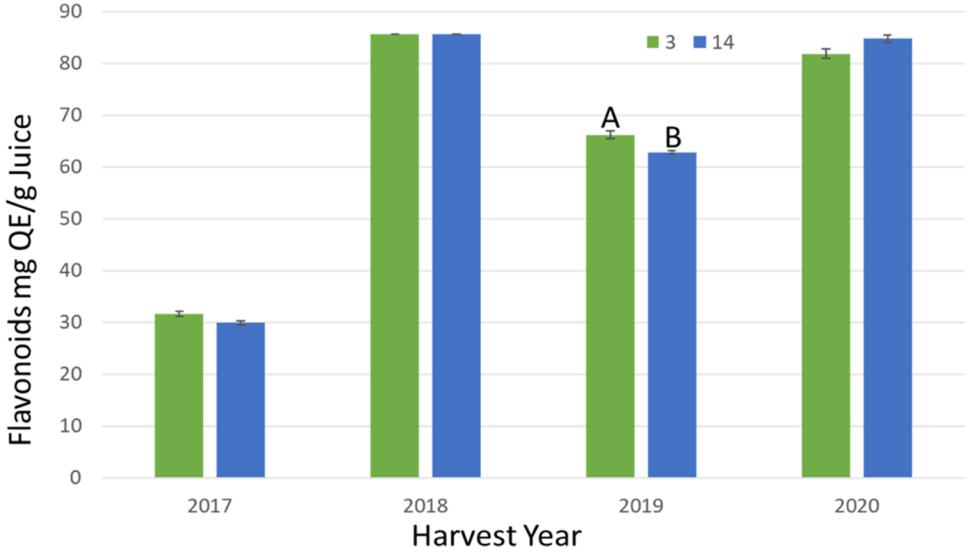

Figure 7. Average flavonoid content of fruit juice from plants between treatments in Plot B from 2017 to 2020. Plants were treated annually with either 3 g of N (green) or 14 g of N (blue). Different letters within harvest year indicate significant differences between treatments ($p \leq 0.5$). Error bars indicate one standard error.

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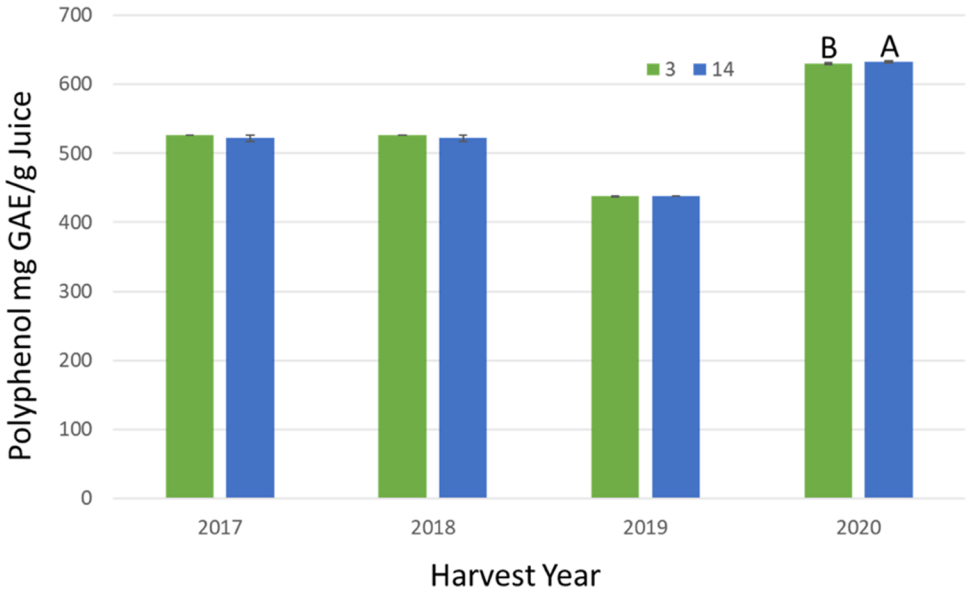

Figure 8. Average polyphenol content of fruit juice from plants between treatments in Plot B from 2017 to 2020. Plants were treated annually with either 3 g of N (green) or 14 g of N (blue). Different letters within harvest year indicate significant differences between treatments ($p \leq 0.5$). Bars without letters indicate no differences in that year. Error bars indicate one standard error.

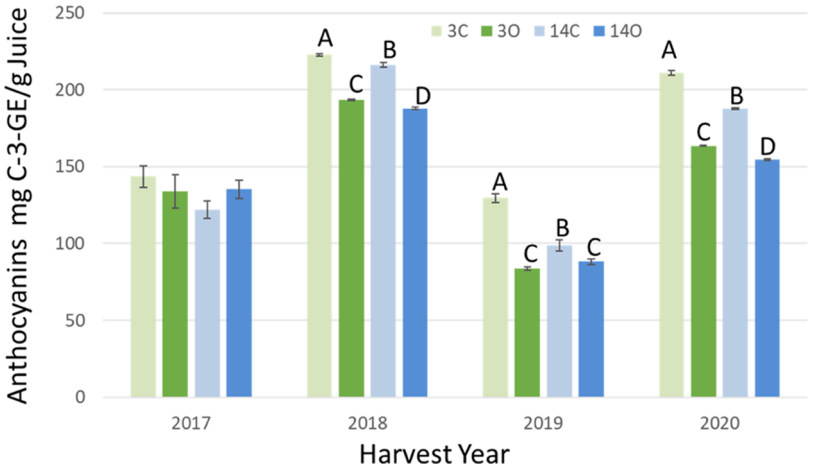

Figure 9. Average anthocyanin content of fruit juice from plants between treatments in Plot C from 2017 to 2020. Nitrogen treatments per plant are as follows: 3O is 3 g of N in organic form, 3C is 3 g of N in conventional form, 14O is 14 g of N in organic form, and 14C is 14 g of N in conventional form. Different letters within harvest year indicate significant differences between treatments ($p \leq 0.5$). Bars without letters indicate no differences in that year. Error bars indicate one standard error.

In 2018, fruit soluble sugar content (°Brix) was measured with an autotemperature compensated refractometer (Fisherbrand HDR-P1, Thermo Fisher Scientific, Bridgewater Township, NJ) after harvest by taking individual juice samples from 10 fruits from the harvest subsample. We have noticed that soluble sugars developing in aronia reach their peak late in August. °Brix values measured at this peak were in the range of 16.0−16.5 for all samples from 2018, 2019, and 2020 harvests. °Brix in 2021 was slightly higher in the range of 18.5−19.0.

Observations for Plot B. Figure 6 depicts the average fruit anthocyanin content from plants in Plot B given 3 or 14 g of N from 2017 to 2020. A nitrogen rate of 3 g had a significantly higher anthocyanin content in 2018 and 2019. A dilution effect from yield is unlikely in 2017 because between 2019 and 2020 there was little difference in yield between those years, and in 2017, between either N rates.

Figure 7 shows the average flavonoid content in fruit juice from plants between treatments in Plot B given 3 or 14 g of N from 2017 to 2020. Differences in flavonoid content were significantly higher only in 2019 with the 3 g N treatment.

Figure 8 shows the average polyphenol content in fruit juice from plants in each treatment in Plot B from 2017 to 2020. No differences in content were seen between 2017 and 2018, but in 2020, fruit from the 14 g N treatment had a slightly higher content compared to 3 g of N.

Observations for Plot C. Figure 9 depicts the average anthocyanin content of fruit juice per year between treatments of nitrogen (N) amount applied in grams (3 or 14 g) as either conventional or organic fertilizer (C or O, respectively).

Comparison of the anthocyanin data presented in Figure 6 shows that, in 2017, anthocyanin content in the juice of fruit was not significantly different between treatment combinations and averaged between 122 and 135 mg APC per g of juice. In 2018,

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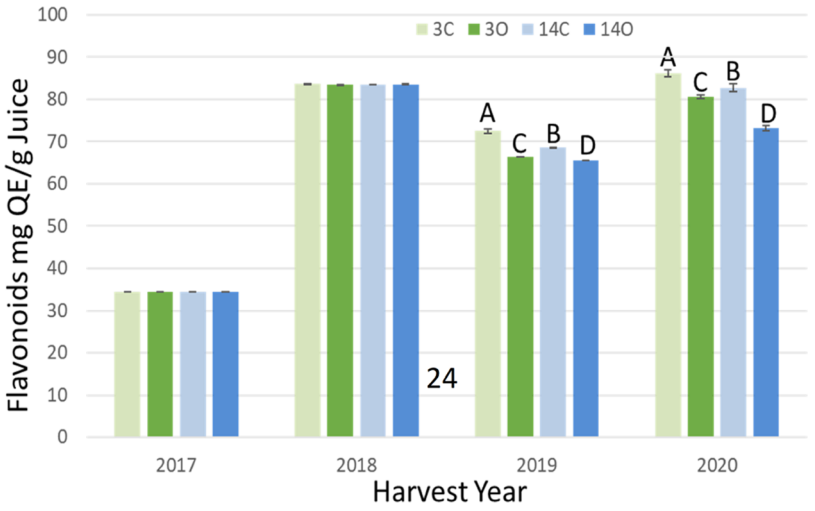

Figure 10. Average flavonoid content of fruit juice of plants between treatments in Plot C from 2017 to 2020. Treatments are as follows: 3O is 3 g of N in organic form, 3C is 3 g of N in conventional form, 14O is 14 g of N in organic form, and 14C is 14 g of N in conventional form. Different letters within harvest year indicate significant differences between treatments ($p \leq 0.5$). Bars without letters indicate no differences in that year. Error bars indicate one standard error.

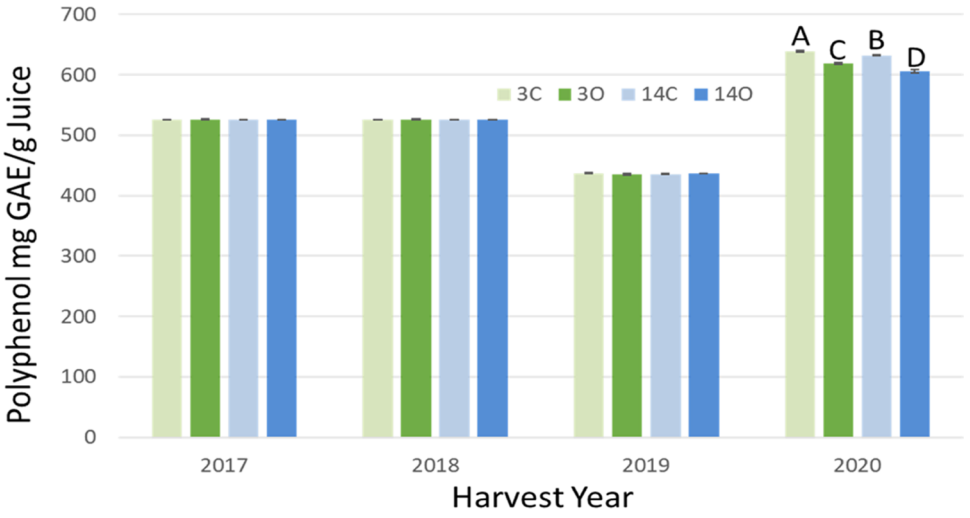

Figure 11. Average polyphenol content of fruit juice of plants between treatments in Plot C from 2017 to 2020. Treatments are as follows: 3O is 3 g of N in organic form, 3C is 3 g of N in conventional form, 14O is 14 g of N in organic form, and 14C is 14 g of N in conventional form. Different letters within harvest year indicate significant differences between treatments ($p \leq 0.5$). Error bars indicate one standard error.

the average anthocyanin content increased over 700 mg g^{-1} juice from 2017. In Plot C (Figure 9), fruit juice from plants given 3 g of conventional N was significantly higher than that of the other treatment combinations, averaging 223 mg of APC per g of juice. The APC content among the other three treatment combinations was lower, decreasing significantly from 14 g of conventional N to 3 g of organic N to 14 g of organic N, respectively. In 2019, the average anthocyanin content dropped from 2018, an average of 105 APC mg g^{-1} juice across all treatments. Although the average anthocyanin content varies significantly each year, those treated with a conventional fertilizer had a higher anthocyanin content within the N rate compared to those treated with an organic fertilizer. Also, a lower N rate had a higher anthocyanin content than a higher N rate. It appears that conventional fertilizers with more available N had a positive effect on anthocyanin production as did less N, which negatively affected the yield. These may be important implications for growers, however. Even though a statistically significant increase in anthocyanin content was noted for the lower N rate, the higher N rate would have produced more fruit, and hence more anthocyanin per hectare. Also, this may dispel some theories that organic fertilizers produce higher quality fruit. As in Plot B, there is unlikely any relationship between yield and anthocyanin content, although interestingly, a similar pattern of juice anthocyanin content exists by harvest year between the two plots. There may be an environmental factor not accounted for in this study regarding this pattern and the differences between harvest years. The rainfall during the growing season of 2018 produced one of the wettest years in Maryland on record.[31]

Figure 10 shows the average flavonoid content of fruit juice from plants between treatments from 2017 to 2020. Although the average flavonoid content varies significantly each year, those treated with 3 g of conventional N produced higher quality fruit than those treated with organic N in 2019 and 2020, but not the 2 years before. When analyzed further, 14 g and conventional (14C) typically produced fruit containing slightly higher anthocyanin concentrations than both 3O and 14O.

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ACS Omega 2023, 8, 4060−4071

Table 3. Analysis of Cyanide Content of Aronia

sample	N rate, g N/bush/year	fertilizer	μmol HCN/100 g juice	mg HCN/kg
aronia juice	0	organic	2.5394131	0.686022
aronia juice	0	organic	3.4065193	0.920271
aronia juice	3	organic	3.7484174	1.012635
aronia juice	7	organic	3.8473879	1.039372
aronia juice	7	organic	3.6494469	0.985898
aronia juice	3	convtl.	3.2448941	0.876608
aronia juice	3	organic	2.2908520	0.618874
aronia juice	14	organic + A	2.4487433	0.661528
aronia juice	14	organic	2.4168144	0.652902
aronia juice	14	convtl.	2.0299298	0.548386
unripe aronia juice	N/A	organic	1.6893700	0.456383
unripe aronia extract of stems	N/A	N/A	11.6545578	3.148479
overripe peach juice	N/A	(store-bought)	1.2318842	0.332794
blueberry juice	N/A	(store-bought)	1.1338013	0.306296
blackberry juice	N/A	(store-bought)	1.6862181	0.455532

In Figure 11, the average polyphenol content in fruit juice of plants between treatments is presented. Although the average polyphenol content does not vary significantly between years from 2017 to 2019, in 2020, those treated with 3 g of conventional N produced higher polyphenols than those treated with organic N.

Anthocyanin, flavonoid, and polyphenol contents in the aronia juice fluctuated in a similar pattern between harvest years. Further study into what may cause these fluctuations in aronia is warranted. A study conducted in Australia on several apple varieties correlated increased air temperatures and global radiation with enhanced synthesis of polyphenols.[32]

Cyanide Content in Aronia Fruit. Cyanides are salts or ester of hydrocyanic acid containing the anion CN^- or the group −CN. This compound is very toxic when it encounters the body. It can replace the heme on the red blood cells in the body, making a strong irreversible bond with the iron in red blood cells. HCN is infamous as a highly poisonous room-temperature liquid, with the lethal dose, LD_{50}, reportedly being in the range of 150−173 ppm (178−206 ppmv) via inhalation for a 30 min exposure time and it can also cause fatalities upon contact with the skin or through ingestion.[33]

Cyanogenic glycosides are compounds that liberate HCN on treatment with dilute acid.[34] These compounds can be naturally found in plants and fruit seeds. Pits of fruits, such as peaches, contain a relatively high concentration of cyanogenic glycosides. However, the concentration is far from that required to display toxic effects. The toxicity rises from the production of hydrocyanic acid (HCN) upon ingestion. Early research has found cyanide to be acutely toxic to aerobic organisms at concentrations generally greater than 0.1−0.3 mg/L, causing death within 96 h.[35] HCN is toxic enough where only 200 mg is a lethal dose for an adult human. An atmosphere containing 200 ppm will result in death within a few minutes.[36]

Cyanide concentration is a parameter of interest to ensure that the fruit is safe for human consumption and must be analyzed for each new fruit introduced to the market. Cyanide concentrations of aronia samples were measured, and there seemed to be no substantial difference regarding treatment, as can be seen in Table 3. Values ranged from 2.83 to 3.85 μmol HCN/100 g juice or 0.55−1.04 mg/kg. Aronia fruit juice samples had an average cyanide content of 0.80 mg HCN/kg. Although these values were higher than that of the unripe sample of aronia and store-purchased peaches, blueberries, and blackberries, these rates were significantly lower than that of other consumed fruits such as various species of passion fruit[37] and the toxic limits established by the EPA. Acute toxicity levels are said to be around concentrations of about 3.7−11.1 μmol/100 g or 1−3 mg/kg body weight in the blood, and 200 mg of cyanide is lethal.[38]

Cyanogenic glycosides are naturally occurring compounds in both fruits and plants. The cyanide content in our fruit samples is low and safe for consumption. The metabolism of cyanogenic glycosides in the body results in the production of hydrogen cyanide gas (HCN). However, HCN readily dissolves in the body, making it available as both hydrogen and cyanide ions. Free cyanide ions in the body are harmful mainly due to their affinity for iron found in the heme complex of blood. Covalent bonding to iron causes oxidation dysfunction due to the inability of the red blood cells to receive and distribute oxygen to various organs of the body system. High concentrations of or prolonged exposure to HCN will result in death.

The cyanide levels in the fruit of mature and immature aronia fruit juice were noticeably lower than that of the stem and peduncle of unripe aronia, measuring 3.15 mg/kg HCN. Using mechanical harvesters or the sales of fresh fruit, stem material may remain with the fruit, and the possibility of human consumption of stem tissue exists. For this reason, an extract of aronia stem was analyzed for cyanogenic glycosides. Although the cyanide levels in the stem and peduncle of unripe aronia were significantly higher than that of the aronia fruit juice, this value pertained to the total cyanide content present in the stem after extraction and whole stems would not be consumed in any regular instance. The partial values that would finally be present in the resultant fruit juice will still be well below the threshold for observing toxicity as was previously seen in Table 2. Therefore, aronia fruit is safe for consumption and no harm would come from harvesting the berries early or including some of the stem in the quicker mass-harvesting procedures.

CONCLUSIONS

Aronia has the potential to be a highly marketable alternative crop. The plant is very hardy and relatively simple to grow, with a low agrichemical input. This research showed that yields in the fruit were dependent on the N rate and the N source applied during and after establishment. Jeppsson (2000)[9] showed that a mix of organic and conventional nitrogen affected yield and fruit quality. This study shows that sustainable N rates can be applied

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ACS Omega 2023, 8, 4060−4071

for optimal yield compared to Jeppsson's rates but suggests that conventional N, which has a quicker release rate than organic N, may not improve the yield but have a slightly positive effect on phytochemical production. The plants may utilize the available N early in the growing season, which most likely supports healthy canopy growth and in turn may support higher photosynthate production. Organic fertilizers are expensive, and no information about the price differential between organic and nonorganic produced aronia is readily available. Apart from blood meal, which was used in this study, other popular organically certified fertilizers include kelp extracts, chicken byproducts, and fish emulsions, which are applied to the soil or by foliar application. None of those were investigated here. Because organic fertilizers are more costly, growers should identify their price point between certified organic and nonorganically grown fruit. Is organic certification worth the cost? For now, organic growers should consider their options based on fertilizer availability. A fertilizer with at least some of its N as readily available, and not solely water-insoluble N or WIN, is recommended. However, more importantly, annual weather conditions such as rainfall, temperature, and global radiation may have a more significant role. These are not something that a farmer can control. Greater yield may translate into greater anthocyanin content per hectare. For instance, a plant yielding 7 kg of fruit will yield on average 3.78 L of juice. There were little to sometimes no differences between the phytochemical content and N treatment (when phytochemicals were tested). This research showed that minimal N rates of 3 g N plant^{-1} year^{-1} could not maintain the yield compared to 14 g plant^{-1} year^{-1} and that moderate rates of N around 14 g N plant^{-1} year^{-1} should be utilized during and after establishment to maintain the vigor and health of plants, including the appropriate soil nutrient amendments. These N rates equate to approximately 36 kg N ha^{-1} given a density of 2600 plants ha^{-1}. Since yield is a large determinant of the total polyphenol production per hectare, use of the proper N rate and ensuring plants have adequate nutrition are vital.

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Notes

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ABBREVIATIONS USED

UV/vis	UV/visible spectrophotometry
μL	microliter
kg	kilogram
m	meter
mL	milliliters
ha	hectare
M	molar
C3GE	cyanidine-3-glucoside equivalents
PP	polyphenols
GAE	gallic acid equivalents
QE	quercetin equivalents
ppm	points per million
A	absorption
Conc.	concentration
N rate	nitrogen rate
NPK ratio	nitrogen/phosphorus/potassium ratio
APC	anthocyanin pigment concentration
Convtl.	conventional (growing)
LD	lethal dose
C3G	cyanidin-3-glucoside
LCMS	liquid chromatography-mass spectrometry

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