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Biochar Influences Phytochemical Concentrations of *Viola cornuta* Flowers

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Abstract: Edible flowers are a rich source of phytochemicals with potential health benefits. Yet, changes in production practices can influence the phytochemical composition of edible flowers. Practices such as the addition of biochar have been used to affect growing media properties as well as to conserve peat resources. However, there is little known about how biochar affects the phytochemical composition of edible flowers. To determine if biochar affects phytochemicals in *Viola cornuta*, four cultivars were subjected to different rates of biochar, with and without fertilizer. At the rate of 10% biochar and without fertilizer application, flower polyphenol and flavonoid concentrations were decreased by 10–20% in two cultivars. However, at 25% biochar, flower polyphenol concentrations varied widely. When fertilizer was added, no effects of biochar were seen. Phytochemical characterization of unfertilized plants further revealed that while increased rates of biochar reduced concentrations of certain antioxidant compounds, these compounds were increased when fertilizer was added. Overall, fertilization can counteract some of the negative effects of biochar on *Viola* cultivars, resulting in higher nutritional quality and an increase in bioactive compounds produced, providing an ability to replace the peat moss with biochar.

Keywords: biochar; *Viola*; peat moss; phytochemicals; phenolic; flavonoid

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1. Introduction

The demand for edible flowers is growing worldwide, not only because of their flavor, color, and odor, but also because of their health benefits [1]. Edible flowers are a rich source of phytochemicals with potential health benefits [2]. Phytochemicals are the bioactive non-nutrient compounds found in fruits, vegetables, whole grains, and other plant foods, responsible for reducing the risk of major chronic diseases, such as cancer, cardiovascular diseases, and obesity [3]. Flavanols, flavones, anthocyanins, and phenolic acids are the most common phytochemicals found in edible flowers [2] and possess antioxidant activity [4]. The antioxidant capacity of plants directly depends upon the levels of anthocyanins and other antioxidant compounds present. Therefore, the higher the anthocyanin content, the greater the antioxidant capacity of the plant [5,6]. In plants, polyphenolic compounds such as flavonoids are responsible for responding to stress caused by unfavorable environmental conditions in adverse climates [7]. Flavonoids, including quercetin and apigenin, possess antibacterial properties and are commonly found in some flowers. These compounds have various functions and actions in the human body but have also been shown to have benefits when added to a balanced diet.

Many production management practices, such as artificial lighting, irrigation, and soil additives, have been known to affect phytochemical production in plants [8]. Among the different soil additives used to improve soil productivity, the one gaining rapid popularity is biochar. Biochar is considered to be a beneficial additive in container production, as it is economically viable and can be produced from different wastes, including forest waste, wheat straw, sugarcane bagasse, and rice hull [9]. They are also responsible for conserving peat moss as they can be replaced as the peat moss substitute in the container production of different crops [10–12]. Biochar has also been shown to increase productivity in plants through increased growth and yields [13,14], which mainly depends on the plants and biochar used. Biochar also possesses phytoremediation properties [15]. These beneficial effects are primarily due to changes in the physiochemical properties of soil [16,17]. Thus, biochar can change the physical attributes of growing media in greenhouse container production when mixed with potting mix [18]. Although biochar is used as an amendment in pot cultivation, it is unable to provide all the essential nutrients for plants as they have comparatively low available nitrogen to plants [19,20]. The nitrogen form present in feedstock can be lost during the pyrolysis process [21]. According to the carbon–nutrient balance hypothesis, the plants produce more carbon-containing secondary compounds when they lack mineral nutrients [22]. Adequate fertilization is therefore needed for the overall growth of the plants. Inadequate nitrogen fertilization leads to the production of non-nitrogen-containing secondary metabolites such as phenolics [22] as a result of nutritive stress. Fertilization can also influence the phytochemical composition of plants depending upon the fertilizer type [23,24].

Viola cornuta is one of the most popular edible flowers produced as bedding plants [25]. Violas are annuals or short-lived perennials that grow 10–15 cm tall and produce 2.5 cm wide flowers [26]. In recent years (2015–2020), sales of *Viola* have increased in the U.S., from approximately USD 95 to 100 million, and at the same time, the number of *Viola* producers also increased from 1714 to 1869 [27,28]. They are one of the most widely used flowers for aesthetic functionality of foods. They are typically used for the decoration of cakes and desserts [29]. They have also gained popularity among food enthusiasts due to their vibrant colors and nutraceutical values [30]. Different compounds such as quercetin, violaxanthin, salicylic acid, flavonoids, tannins, alkaloids, auroxanthin, and flavoxanthin were also found in *Viola* flowers [31,32]. Several studies have been conducted to determine the phytochemical compounds possessing antioxidant abilities in *Viola* [30,33,34].

Most of the current research on biochar's impacts on plant production has focused on quality, stress tolerance, and yields. Very few studies have examined the influence of biochar rates on phytochemical production in plants. Furthermore, there is no research available on how the phytochemical compositions of *Viola* cultivars are affected by biochar application rates. We hypothesized that the application of biochar and fertilization can increase the phytochemical production of *Viola*. Therefore, the objective of this study was to evaluate the effects of various biochar applications on the phytochemical concentration of *Viola* under nutritional stress. This manuscript is a companion paper to Regmi et al. 2022, [11] which studied the physiological effects of fertilization and biochar on *Viola*.

2. Materials and Methods

2.1. Growing Conditions and Planting Materials

Two container experiments were conducted from 1 November 2019 to 12 December (expt. 1) and 10 September 2020, to 12 January 2021 (expt. 2) in the Horticulture Gardens and Greenhouse Complex of Texas Tech University, Lubbock, TX (latitude 33.584790 and longitude −101.883650). Growing media were prepared by mixing commercial potting mixture Metro-Mix 852 (35% Canadian Sphagnum peatmoss, 55% composted pine bark, 10% coarse perlite, and dolomitic limestone; Sungro Horticulture, Agawam, MA, USA) with three designated rates of hardwood biochar (Wakefield Biochar, Columbia, MO, USA) at ratios of 90:10, 75:25, and 50:50 (% wt. of potting mixture: % wt. of biochar). Growing media pH ranged from 6.32 to 7.17 in expt. 1 and from 6.28 to 7.10 in expt. 2. Electrical conductivity (EC) ranged from 0.28 to 1.40 dS/m in expt. 1 and 0.12 to 0.98 dS/m in expt. 2, as described by Regmi et al. [11]. There were not significant differences between EC in either trial but pHs were greater at higher biochar rates. The chemical properties of hardwood biochar is described in Parkash et al. [35]; the same commercial biochar sources were used in both studies. Briefly, the feedstock was oak, organic matter comprised 82% of total mass, while NPK were 0.64%, 3.52 mg/kg, and 2960 mg/kg, respectively [35]. Seedling plant materials were sourced from Desert Rose Plant Farm (Lubbock, TX, USA) under Eason Horticultural Resources (Covington, KY, USA) for both experiments. As flower color has a large impact on phytochemical composition in flowers [30], three different cultivars of *Viola cornuta* with different petal colors were selected in each experiment (Deep Blue (DB), Penny Yellow (PY), and All-Season Mix (ASM)/Johnny Jump Up (JJ)). DB, PY, and one mixed tray of ASM cultivar were used in expt. 1. Due to non-availability from suppliers, ASM was replaced with JJ in expt. 2.

Viola seedlings were transplanted into prepared 10×10 cm^2 pots with 12 cm depth, filled with described biochar treatments. They were then arranged in a completely randomized design in both trials. The *Viola* plants were grown without fertilizer in the first experiment, whereas 20 mL of water-soluble Jack's fertilizer ($N{:}P_2O_5{:}K_2O$-20:20:20; JR Peters Inc., Allentown, PA, USA) was applied weekly in the second experiment at a 0.50 g/L H_2O rate, starting seven weeks after planting. Treatments were replicated 10 times in expt. 1 and 12 times in expt. 2, for a total of 120 and 144 plants, set up in a completely randomized design. The average greenhouse air temperature was 30 °C/°C (day/night) for both experiments. Plants were irrigated with reverse osmosis water as needed each week. Fully expanded flowers were harvested weekly and stored at −80 °C for phytochemical analyses.

2.2. Extraction Methods

To extract water-soluble polyphenols from *Viola* flowers, a water decoction method was used as described by Li et al. [36], with some modifications. Flowers were dried, then ground to a fine powder using a mortar and pestle. Next, 0.125 g of the powdered sample was added to 2.5 mL of distilled water and placed in a water bath at 100 °C for 30 min. Samples were left to cool at room temperature before being centrifuged at 3500× *g* for 10 min (Eppendorf Centrifuge 5430, Enfield, CT, USA). The obtained supernatant solution was used to determine the total polyphenolic and flavonoid concentration in mg gallic acid equivalent per gram (mg GAE/g DW) and mg quercetin equivalent per gram (mg QE/g DW) of dried sample, respectively.

2.3. Total Polyphenol Analysis

To determine the total polyphenolic concentration in *Viola* flowers, the Folin–Ciocalteu method was used as described by Singleton et al. [37], with modifications by Li et al. [36]. The standards and samples were also prepared according to Li et al. [36] Briefly, gallic acid was used as the standard curve solution for polyphenol analysis. For the standard curve, 50 µL of each standard solution was pipetted into a 10 mL glass test tube and mixed with 0.395 mL water and 25 µL Folin–Ciocalteu reagent (Millipore Sigma, Billerica, MA, USA). For the sample extracts, 50 µL of the extracts were added to 0.25 mL of 0.2 N Folin–Ciocalteu reagent in a 10 mL test tube. The solutions were then left at room temperature for 4 min. Next, 75 µL of a saturated sodium carbonate solution was added to each test tube. Each tube was the thoroughly mixed and left at 20 °C (room temperature) for 2 h. Finally, 200 µL of the prepared samples were pipetted into different wells in an optically clear 98-well microplate, and then placed in a microplate reader (SpectraMax iD3, Molecular Devices, San Jose, CA, USA) for absorbance determination. Absorbance was measured at 760 nm to determine polyphenol concentration.

2.4. Total Flavonoid Analysis

To determine the total flavonoid concentration of the flower, the Aluminum Chloride Colorimetric Method was used as described by Chang et al. [38], with some minor modifications. Quercetin was used to prepare the standard curve solution for total flavonoid analysis. Standards ranging from 25–800 µg/mL were prepared according to Chang et al. [38]. For the standard curve, 0.5 mL of each standard solution was mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water, then incubated at room temperature for 30 min. For the sample, 0.5 mL of sample supernatant was mixed with 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 3.8 mL of ethanol. Next, 50 µL of this solution was transferred to a microplate with 150 µL of distilled water (dilution factor of 4). The microplate was read at 410 nm with the microplate reader. The flavonoids were reported as mg of quercetin equivalent/dry weight g sample (mg QE/g DW).

2.5. Sample Characterization via HPLC-MS

Ground, dried samples of *Viola* sp. cv. DB were sent to the Texas Tech University Center for Biotechnology and Genomics for characterization and relative compound concentration comparison via HPLC-MS, according to methods described by Koike et al. [39] Briefly, dried flower samples were extracted in 20 mL of methanol containing 0.5% trifluoroacetic acid, then filtered and reextracted in the acidified methanol solution. These extracts were evaporated and the dried sample was then redissolved in water. The samples were purified, eluted, lyophilized, and dissolved in 1 mL of 20% aqueous methanol and then filtered for HPLC-MS analysis. The control samples were then compared to the 10% and 25% biochar-treated DB flower samples from expt. 1 to determine the relative change in compounds of interest. This characterization was repeated for expt. 2, but flowers from the 50% biochar treatment were also included.

2.6. Statistical Analysis

Container experiments were organized in a completely randomized design, with biochar and cultivar as different treatment levels. Differences between biochar rates and cultivars were determined using analysis of variance, and interactions between treatments and cultivars were identified. Significance levels were set at $p \leq 0.05$ and differences among means were determined using a Tukey's HSD test.

3. Results

3.1. Bioactive Compounds

In expt. 1, there was an overall interaction effect between biochar and cultivar on the total phenolic concentration of *Viola* (Table 1; $P\ b \times c \leq 0.005$). The highest concentration of polyphenols was found in DB in the control treatment. When comparing cultivars, DB had 26% and 20% more polyphenols than PY and ASM, respectively. Otherwise, polyphenols varied by treatment and cultivar and no specific trends were identified. Biochar rates did not impact polyphenols in different cultivars significantly. However, a slight reduction of numerical values of DB polyphenol concentration can be found at higher biochar rates (Table 1). Conversely, in the fertilizer experiment (expt. 2), no interactions were observed between treatments and cultivar, and the only significant effects on polyphenols were between cultivars (Table 1). However, polyphenols increased numerically at higher biochar rates compared to control. Within cultivars, polyphenols showed non-significant increases when biochar was added. For example, polyphenols of PY increased by approximately 10% in 25% biochar rates, but decreased in the 10% biochar rate by 21%. Alternatively, polyphenols in ASM increased at both 10 and 25% biochar by 49 and 41%, respectively. The addition of 50% biochar specifically impacted polyphenol concentrations of *Viola*. Polyphenols increased among all cultivars of *Viola*, ranging from 1% in PY at 10% biochar to 55% in JJ at 50% biochar rates. When comparing cultivars alone, DB had 229% and 52% more polyphenols than PY and JJ, respectively. Overall, the highest total phenolic concentration was found in DB flowers, followed by JJ, with the lowest phenolic concentration in PY. In both experiments, DB had higher polyphenol concentrations as compared to other cultivars.

Table 1. Average total phenolic concentration and flavonoid concentration of *Viola* flowers.

Cultivar	Biochar Rate	Total Water-Soluble Polyphenols (mg GAE/g Sample)	Change in Polyphenols (% of Control)	Water-Soluble Flavonoid (mg Quercetin Equivalent/g Sample)	Change in Flavonoids (% of Control)
Expt. 1					
Deep Blue	**Control**	359.46 a		23.35 ab	
	10	286.92 ab	−20.18	20.12 bcd	−13.80
	25	330.96 a	−7.93	20.98 bc	−10.13
	50	-		-	
Penny Yellow	**Control**	266.78 ab		15.05 cd	
	10	210.83 b	−20.97	13.41 d	−10.88
	25	293.41 ab	9.98	16.46 bcd	9.34
	50	-		-	
All Season Mix	**Control**	198.44 b		19.83 bcd	
	10	311.56 ab	57.00	29.60 a	49.25
	25	297.74 ab	50.04	28.05 a	41.44
	50	-		-	
F-ratio		5.21		13.88	
df		8, 18		8, 16	
P cultivar		*0.005*		*<0.001*	
P biochar rate		0.122		0.130	
P B × C		*0.005*		*0.003*	
Expt. 2					
Deep Blue	**Control**	276.36 A	-	19.40 A	-
	10	297.18 A	7.53	24.17 A	24.59
	25	288.18 A	4.28	18.72 A	−3.51
	50	284.27 A	2.86	20.53 A	5.82
Penny Yellow	**Control**	81.58 C	-	15.04 B	-
	10	82.92 C	1.64	13.41 B	−10.84
	25	85.36 C	4.63	15.20 B	1.06
	50	98.90 C	21.23	20.78 B	38.16
Johnny Jump Up	**Control**	146.38 B	-	16.58 B	-
	10	192.54 B	31.53	15.32 B	−7.60
	25	183.35 B	25.26	13.43 B	−19.00
	50	226.98 B	55.06	18.14 B	9.41
F-ratio		23.98		1.72	
df		11, 24		11, 24	
P cultivar		*<0.001*		*0.019*	
P biochar rate		0.135		0.282	
P B × C		0.485		0.498	

Mean separations were determined via Tukey's HSD tests. Lowercase letters indicate significant interaction effects between cultivar and biochar rate. Uppercase letters indicate significant differences between biochar rates. P B × C indicates interaction between biochar rates and cultivars. Italicized numbers indicate significance at $p \leq 0.05$.

There was also an interaction effect between biochar and cultivar on the flavonoid concentration of *Viola* (Table 1; P b × c ≤ 0.003) in expt. 1. Where the highest flavonoid concentrations were found in ASM with 10% and 25% biochar rate, the lowest flavonoid concentrations were found in PY with the 10% biochar rate. As with the polyphenol results, cultivar also had a significant impact on flavonoid concentration in *Viola* flowers (Table 1; P cv < 0.001), with ASM having 20% and 72% more flavonoids than DB and PY, respectively. In the blue/purple pigmented DB cultivar, increased biochar reduced flavonoids at both 10% and 25% rates numerically. However, in the yellow pigmented PY, flavonoid was reduced at 10% biochar but increased by approximately 9% at 25% rates of biochar. Moreover, the greatest percent increases in flavonoids were found in the ASM at 10% and 25% biochar rates, by 49% and 41%, respectively. After fertilization, the effects of biochar were not seen on flavonoids in violas. However, cultivars did show differences in flavonoid concentrations, with DB having higher concentrations than PY and JJ, with increases of 29% and 30%, respectively. Although no significant effect was seen on the flavonoid concentration, the biochar rate numerically increased the flavonoid concentration of *Viola* flowers up to 38% in the higher rates of biochar (50% biochar), depending upon the cultivar.

3.2. Sample Characterization via HPLC-MS

The initial characterization of control samples found over 4500 compounds and metabolites in DB flowers. More than 500 individual plant compounds were found in each of the dried samples during this characterization. A comparative analysis was performed to determine how the sample compositions varied from the control. Without fertilization, two distinct flavonoids, quercetin-3β-D-glucoside (quercetin) and myricetin 3-O-β-D-galactopyranoside (myricetin), were found in significantly different concentrations when comparing the control to biochar-treated plants (Table 2). Concentrations of both compounds were reduced as compared to the control, with the greatest decrease seen in 25% biochar-treated violas. Each of these polyphenolic compounds decreased in concentration as biochar treatment rates increased. Alternately, ethyl ascorbic acid, a derivative of vitamin C, increased in 10% biochar-treated *Viola* plants without fertilizer. Conversely, in expt. 2, when fertilization was applied, all these compounds increased in biochar-amended treatments. Altogether, concentrations of 16 distinct phenolic compounds differed significantly in biochar-treated plants as compared to the control (Table 3). Flavonoids, quercetin, myricetin (including dihydromyricetin), and xanthorhamnin increased in flowers of 10%, 25%, and 50% biochar-treated plants compared to the control. However, myricetin increased in 25% biochar-treated plants, but then declined in the 50% biochar treatment. Rutin also increased in 50% biochar-treated plants by 0.6 log 2-fold, whereas there was only a 0.27 log 2-fold increase in 10% biochar. Certain flavones, isovitexin and pteridine, decreased in 10% and 25% biochar, but luteolin increased only in 50% biochar-treated DB flowers. For the flavanones, eriodyctyol increased in 25% and eriocitrin and naringenin increased in 50% biochar-treated plants. The phenolic acid, gallic acid, only increased in 50% biochar-treated DB flowers.

Table 2. Comparative change in select phytochemicals in *Viola* flowers with added biochar in Experiment 1.

	10% Biochar			25% Biochar		
Class	**Name**	**Log2 Fold Change**	***p*-Value**	**Name**	**Log2 Fold Change**	***p*-Value**
Flavonoid	Quercetin-3β-D-glucoside	−0.52	0.039	Quercetin-3β-D-glucoside	−2.70	0.029
	myricetin 3-O-beta-D-galactopyranoside	−0.90	0.020	myricetin 3-O-beta-D-galactopyranoside	−1.86	0.006
Vitamin	2-O-Ethyl ascorbic acid	2.83	0.051			

Log 2-Fold change was compared to the control.

Table 3. Comparative change in select phytochemicals in *Viola* flowers with added biochar and fertilizer in Experiment 2.

	10% Biochar			25% Biochar			50% Biochar		
Class	**Name**	**Log2 Fold Change**	***p*-Value**	**Name**	**Log2 Fold Change**	***p*-Value**	**Name**	**Log2 Fold Change**	***p*-Value**
Flavonoid	Rutin	0.27	0.008	Quercetin-3β-D-glucoside	1.26	0.004	Rutin	0.60	0.039
	Dihydromyricetin	0.65	0.001	myricetin 3-O-beta-D-galactopyranoside	1.03	0.005	Quercetin-3β-D-glucoside	1.40	0.037
	-	-	-	-	-	-	myricetin 3-O-beta-D-galactopyranoside	0.88	0.001
	-	-	-	-	-	-	Xanthorhamnin	0.32	0.011
Flavones	Isovitexin	−0.54	0.048	Isovitexin	−1.16	0.025	Luteolin	1.22	0.049
	Pteridine	−0.92	0.008	-	-	-	-	-	-
Flavanone				Eriodictyol	1.14	0.005	Naringenin	0.56	0.008
	-	-	-	-	-	-	Eriocitrin	1.29	0.003
Phenolic acid	-	-	-	-	-	-	Gallic acid	0.83	0.019

Log 2-Fold change was compared to the control.

4. Discussion

4.1. Impacts of Biochar on Flower Phytochemical Composition (Total Polyphenolics and Flavonoids)

Biochar has been known to affect many biochemical and physiological aspects of plants [13,40,41] in these experiments. Biochar affects the pathway of secondary metabolites by the regulation of genes [42]. The phytochemical composition of *Viola* did not show any specific trend with the application of biochar. Though they were not statistically significant, a slight influence on phytochemical composition was found in biochar rates among both experiments. Because of sample volume, phytochemical analysis cannot be replicated more, which has increased the variability among the samples. However, across both experiments and all cultivars, polyphenol concentrations ranged from 80–360 mg GAE/g DW, which is consistent with findings by Skowyra et al. [43]. It should be noted that the concentrations of polyphenols in unfertilized violas were, on average, greater than in fertilized plants, but the effects of biochar rates were diminished after fertilization. This may be due to stress caused by nutrient deficiency, as plant stress induces production of carbon-containing compounds and secondary metabolites such as phenolics [22,24]. In unfertilized plants, biochar altered the polyphenol concentrations in flowers, which varied by cultivar. However, when plants were fertilized, biochar did not impact polyphenol concentrations; only cultivars showed significant impacts. Because polyphenols differed among cultivars across both experiments, it is likely that pigments associated with different cultivars are the primary factor dictating polyphenol concentrations [44–46]. In expt. 1, each cultivar showed a different phytochemical response to biochar rates. These findings may be due to the different levels of tolerance each cultivar has to the different rates of biochar when under nutrient stress [11], as well as changes in plant metabolism to produce more carbon compounds [22] rather than nitrogen compounds due to nutrient deficiency. However, biochar rate significantly affected the plant nutrient contents, mainly nitrogen and phosphorous [11]. Nitrogen levels in trial 1 were between 1.58–2.49% and in trial 2 they were 2.36–3.64%. Recommended N levels for violas are between 2.5–4.5% [47]. In trial 2, these levels are within the acceptable range, as expected. K levels in plant tissues were between 2.86–3.78% in the first trial and 1.41–3.03% in the second trial. These are mostly within the acceptable levels of K for violas as specified by Owen et al. [47,48], who recommend K levels between 2.5–5.0%. Furthermore, when K is abundant in substrates, there is generally no effect on plants due to luxury consumption. While there are cases where excess K can cause antagonism of other nutrients such as Mg and Ca, this is not evident in our trials, as evidenced by the nutritional profile of plant tissues. For Mg, trial 1 plants were within 0.67–0.94% and 0.83–1.11% for trial 2. Recommended Mg levels are between 0.25 and 0.75% for violas, which does indicate that plant tissues are over this threshold in these studies. However, Mg beyond these levels tends to be stored in the vacuole and high concentrations have primarily resulted in negative responses under drought stress according to Marschner [49], which was not induced in these studies. In general, excessive Mg causes improvements in nutritional quality of plants, but it can cause Ca deficiencies due to antagonism related to plant uptake. This is not the case in these studies, as Ca in plants in trial 1 were 0.99–1.67% and in trial 2 they were 1.38–2.28%. Owen et al. [47] recommend Ca levels between 0.6–3.0% for violas, which indicates that plants were not suffering from deficiencies in either trial. Depending upon the cultivar, nitrogen content of plant tissue was increased in lower biochar rates (10 and 25%), but decreased when higher biochar rates were used without fertilization [11]. Likewise, phosphorous content in plant tissue was reduced at 50% biochar rate when fertilization was not applied. Biochar also has a high sorption capacity and essential elements will bind strongly with biochar, which can lead to reduced availability of certain nutrients such as nitrogen [50]. Nutrient stress and fertilization form can affect antioxidant and phytochemical production within plants [8]. For example, Fallavo et al. [51] found that nitrate fertilizers can increase flavonoids compared to ammonium fertilizers in *Brassica juncea*. However, in our experiment when fertilizer was added, the effects of biochar were no longer significant. Our findings conflict with a study by Phares et al. [52] who found that fertilization increased phenolic compounds in cowpea. However, the fertilizer applied in their study was phosphorous fertilizer and the application rate were also different. Yet, it should be noted that little to no research has examined how biochar affects phenolic compounds in flowers. This illustrates the importance of determining how phytochemical compounds of flowers are affected by biochar. In expt. 1, control and 25% biochar rates in DB cultivars had the highest polyphenol concentrations. Overall, the 10% biochar rate had lower polyphenols in DB and PY cultivars, but the highest polyphenols were in ASM. This is likely because ASM was composed of multiple colors of flowers (white, yellow, and orange). The ranges of total polyphenolics found by Skowyra at al. [43] were between 120 and 466 mg GAE/g DW in different colored flower petals of *Viola,* which are similar to our range as well. They also found higher amounts of polyphenols in violet and red flower petals followed by yellow flower petals, which was true in our study as well. This shows that more polyphenols are found in deeply pigmented flowers. Vukics et al. [53] also found that deeply colored flowers have higher polyphenol contents compared to light colored flowers. The dark blue/violet pigments of *Viola* spp. had higher amounts of polyphenols in the form of anthocyanin when compared with light-colored *Viola* spp. [54]. This is likely the reason for higher polyphenols in DB flowers compared to PY and ASM.

Like polyphenols, flavonoids were affected differently based on biochar rate and cultivar. ASM had the greatest flavonoid concentration, as this producer's blend contained flowers of multiple colors. Flavonoids ranged from 13–30 mg QE/g DW of sample for this study, which is similar to the ranges found by Fernandes et al. [55] on different colored petals of *Viola* flowers. The plants studied by Fernandes were also a different species of *Viola*, which could explain some of the differences found [55]. Furthermore, the different extraction methods and standards used could account for the deviations, as different extraction methods can impact detection of phenolic compounds [43]. Yet, significant differences were found between treatments and cultivars regardless of the extraction method used. These results showed that the mixed color flowers had higher flavonoid concentrations, as they have mixtures of yellow-, white- and orange-colored petals of *Viola*. The mixture of white flowers may have contributed to the higher flavonoid concentration in them, according to findings by Fernandes et al. [30], in which flavonoid concentrations in white petals were greater than that of red- and yellow-colored petals of *Viola* flowers. Vukics et al. [56] also found that the flavonoid concentration was greater in yellow-colored petals of *Viola* cultivars. Interestingly, there have been limited studies on the phytochemical concentrations of flowers of different colors when combined for analysis. Because most edible violas are sold in packages with multiple flower color combinations, they are likely consumed in this manner. Therefore, understanding how phenolic compounds in a mixed grouping of edible flowers (growers mix (ASM)) were affected by biochar and fertilization is also relevant.

Biochar is considered to be an organic material [57] and is known to improve crop productivity by increasing the water and nutrient holding capacity of the soil [58]. In expt. 1, when only biochar was applied, higher biochar rates (50% biochar) were detrimental for *Viola* plants [11]. But when fertilizer was added in the expt. 2, no differences in polyphenols and flavonoids of *Viola* flowers were seen between biochar rates. In experiments where biochar-only substrates were used, biochar negatively affected ornamental plant growth and productivity, which may be due to the change in the physical and chemical properties of the growing media [18]. In a previously published paper, Regmi et al. [11] found that the physiological impacts of biochar at higher rates (~50%) had negative effects on plant growth and overall performance. Although polyphenols and flavonoids were not significantly affected by biochar rate in fertilized plants, polyphenol and flavonoid concentration tended to be higher in biochar treatments when compared to the control in the fertilized experiment. While this was not consistent across cultivars, biochar does seem to have a slight influence. Interestingly, fertilization did not uniformly increase phytochemical concentration across cultivars, which conflicts with results found by other researchers. For example, addition of biochar with fertilization increased the phenolic as well as flavonoid concentration in cowpea [52]. Similarly, the addition of foliar fertilization increased the carotenoid concentration in flowers of *Calendula officinalis* [59]. Only cultivar significantly affected polyphenol and flavonoid concentrations in expt. 2, where DB had greater polyphenols and flavonoids than PY and JJ. It should also be noted that concentrations of polyphenols and flavonoids were greater in expt. 1 compared to expt. 2. According to the carbon/nitrogen balance hypothesis, when nitrogen is unavailable to plants, the metabolism changes to induce the production of carbon-containing compounds and secondary metabolites such as phenolics [22].

4.2. Characterization of Viola Extracts

During sample characterization, quercetin and myricetin were found in extracts of *Viola*. Myricetin and quercetin are flavonols possessing high antioxidant activity [60] and are responsible for reducing oxidative stress and are beneficial in human diets [61]. Of these, quercetin and its metabolites were the most abundant flavonoids found in *Viola* [31]. Several researchers previously established that quercetin and myricetin can be found in extracts of *Viola*, which supports the results found in these experiments [62,63]. A more in-depth sample characterization of unfertilized plants showed that quercetin and myricetin, specifically, decreased in higher rates of biochar. Viger et al. [42] also found that quercetin was reduced in Arabidopsis thaliana when treated with added biochar, which was found true to our study. However, compounds such as quercetin, myricetin, and rutin were increased when the plants were fertilized in expt. 2. Moreover, different flavones, such as isovitexin and pteridine, decreased, but luteolin increased in fertilized *Viola* plants. The varied results among these experiments may be due to the fertilization, which is known to influence the phytochemical composition of plants. Ahmadi et al. [64] found that fertilization can increase the total phenolic and flavonoid concentration in Echinacea purpurea. This increase is likely due to impacts of stress on phenol synthesis pathways. The Shikimic acid pathway is responsible for the synthesis of phenolic compounds in plants, and can be influenced by different biotic and abiotic stressors [65]. In this study, biochar acted as a stressor at higher rates, as evidenced by the effects on growth and biomass [11]. We saw higher corresponding concentrations of polyphenols at these higher rates of biochar as well (Table 3). Biochar is known to influence the amino acid (phenylalanine) concentration of plants [66], which may affect phenol compound synthesis, as they are the pre-cursor of phenols in the Shikimic acid pathway [65]. Różyło et al. [67] found that adding biochar with sewage sludge can also impact phenolic and flavonoid concentration in wheat, but no obvious trend was seen in phytochemical concentration. This was similar to findings in this study, where we did not find any significant trend in the change of specific phytochemicals in *Viola*. However, it can be inferred that either flavonoid composition changed, resulting in a shift from quercetins to other compounds, or biochar influenced metabolite or phenolic compound production in *Viola*. Further research is needed to determine the specific change in biochar ratios and different phytochemicals to fully establish the mode of action. Overall, our findings show that biochar can influence the phytochemical composition in edible flowers, which can then be used to enhance the phytochemicals in plant products and human diets. At the same time, the usage of peat moss can be reduced by replacing it with biochar, which accounts for the sustainable use of peatmoss. However, further research is necessary to fully understand the interaction between biochar and phytochemicals of *Viola*.

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