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Impacts of Climate Warming on Nutritional Quality and Soil Bacterial Communities of Wild Blueberries

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An Abstract of the Dissertation Presented
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Under anthropogenic global climate change associated with greenhouse gas emissions, the mean surface temperature of the earth has increased by approximately 1 ^{o}C since the industrial age and is projected to rise by 2.6 to 4.6 ^{o}C by 2100. Wild blueberry fields in Maine are experiencing unprecedented warming, which may affect the fruit flavor and nutritional quality, soil health and microbial community, and consequently impact yield, human nutrition and the economic well-being of wild blueberry producers. It is therefore important to assess the impact of climate warming on fruit crop nutritional quality and soil microbial community, and test potential strategies to mitigate the potential negative effects. Additionally, the genetic diversity of wild blueberries has not been well-studied, which will influence their response to climate change. The objectives of this research were to: 1) quantify the impact of warming on the nutritional qualities of wild blueberries, 2) assess the effect of biochar-compost mix and mulching on mitigating the negative impact of warming on fruit nutritional qualities, 3)

investigate the impact of warming on the wild blueberry soil bacterial communities, and 4) evaluate the genetic diversity and structure of wild blueberry populations in managed and unmanaged fields using single nucleotide polymorphic markers.

First, I examined the nutritional quality (i.e., berry minerals, total soluble protein, total soluble solids, soluble sugars, anthocyanin) of wild blueberries grown under three temperature conditions: (1) ambient conditions, (2) passive open-top heating that elevated average temperatures by 1.2 ^{o}C, (3) active open-top heating that elevated average temperatures by 3.3 ^{o}C. Our results suggest that total soluble solids, fructose, total soluble sugars, and total soluble protein concentrations decreased as temperature increased. These changes need to be further studied to determine if they would impact consumer preference or human nutrition, and if so, mitigation techniques should be developed and tested.

Second, I assessed the potential of two mitigation strategies, biochar-compost mix (BCM) and mulching for mitigating the detrimental impacts of warming on the nutritional quality of wild blueberries. The negative effect of warming on total soluble protein and total soluble solids, as well as demonstrating strong negative effects on secondary metabolites, antioxidants, organic acids, and phenolic components was confirmed. The mitigation strategies did not reduce the negative effect of warming on wild blueberry fruit quality except for potassium and magnesium mineral concentrations. The application rate of the biochar-compost mix and mulching currently used may not be sufficient to mitigate the negative effect of warming on berry nutritional quality.

Third, I studied how bacterial communities respond to warming during the growing season, using the passive and active open-top chambers to simulate climate warming scenarios in wild blueberry fields. Overall, soil bacteria diversity and richness (June, July, and August data

combined) under the warming (passive and active) treatments and ambient controls did not show significant differences after experimental warming for two years. However, significantly higher bacterial evenness and diversity under warming treatments were found in the early growing season (June). The increased bacteria evenness and diversity under warming treatments in June could be related to advanced plant phenology, suggesting a future shift of seasonal dynamics in bacterial activity under global warming.

Last but not the least, I evaluated the genetic variation of two wild blueberry species across four fields in Maine using single nucleotide polymorphic markers. Most of the wild blueberry plants were genetically related, regardless of the region. Overall, no distinct genetic differentiation and no difference in genetic diversity were found between managed and unmanaged fields.

This study quantified the genetic variation of wild blueberries within and among fields and provided some insights about the impact of warming on wild blueberry nutritional quality and soil microbial communities. Further studies could be done to determine if changes in nutritional quality would impact consumer preference and human nutrition, and if longer term warming will change soil microbial communities. Techniques to mitigate the negative effects of warming on wild blueberry nutritional quality should be developed and tested.

DEDICATION

I dedicate this dissertation to my parents, family, and friends, who supported, prayed, and motivated me throughout my doctoral studies.

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I would like to thank my advisor, Dr. Yong-Jiang Zhang, who has been a great mentor and has allowed me to learn and grow during my doctoral degree at the University of Maine. I am grateful to my advisory committee members, Dr. Gregory Porter, Dr. Jianjun Hao, Dr. José Meirelles, and Dr. Lily Calderwood, for their valuable guidance, feedback, and support that kept me productive through graduate school. I appreciate every member of Zhang's lab, past and present, for the teamwork that has helped me thrive. Thank you to our collaborators, Dr. Brian Perkins, Dr. Suzanne Ishaq, Dr. Bode Olukolu, Dr. Han Tan, Bruce Hall, and Travis. Also, my sincere appreciate goes to Trish Costello, Perry Killian, Dr. Ann Dieffenbacher-Krall, Dr. Rachel Fowler, Dr. Julia McGuire and Dr. Farahad Dastoor for their support during my with teaching assistantship position. I appreciate the Office of International Programs at UMaine, particularly Sarah Joughin and Mireille Le Gal, for their constant support and assistance as I navigated through the program. I appreciate my parents, siblings, mentors and friends (Kingsley Maduagwu, Emmerson Chivhenge, Kingsley Wiafe-Kwakye, Samuel Akomea-Frempong) for their support and prayers. Most importantly, I sincerely thank my beautiful wife, Esther Alaba, and son, Emmanuel Alaba, for their patience, sacrifice, and amazing support that brought me joy and strength throughout my studies. I could not have achieved this if not for your support. To God be the Glory.

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CHAPTER 1

WILL CLIMATE WARMING REDUCE THE NUTRITIONAL QUALITY OF WILD BLUEBERRIES?

1.1 Abstract

Anthropogenic climate change will potentially affect the nutritional quality of perennial crops, which has not been well-investigated. The wild blueberry is a perennial crop of cultural and economic importance, known for its health-promoting properties. Wild blueberry fields in Maine are experiencing unprecedented warming, which may affect the fruit flavor and nutritional quality and consequently impact human nutrition and the economic well-being of Maine wild blueberry producers at all scales of production. I examined the nutritional quality (i.e., berry minerals, total soluble protein, total soluble solids, soluble sugars, anthocyanin) of wild blueberries grown under three temperature conditions: (1) active open-top heating that elevated average temperatures by 3.3 ^{o}C, (2) passive open-top heating that elevated average temperatures by 1.2 ^{o}C, and (3) ambient control conditions. I found that total soluble solids, fructose, total soluble sugars, and total soluble protein concentrations decreased as temperature increased. In contrast, anthocyanin, total flavonoid, and phenolic concentrations did not differ significantly among treatments. Sugars (fructose and glucose) were positively associated with total soluble solids under ambient and passive warming treatments, whereas no association was found under active warming. Warming also weakened associations among other chemical compounds. Therefore, warming may change associations of metabolites and make total soluble solids measured by portable refractometer not a good indicator of sugars. In conclusion, our results suggest that future global warming may reduce the nutritional value and, consequently, the

perception by consumers of wild blueberries. These changes need to be further studied to determine if they would impact consumer preference or human nutrition, and if so, mitigation techniques should be developed and evaluated.

1.2 Introduction

Under anthropogenic global climate change associated with greenhouse gas emissions, the mean surface temperature of the earth has increased by approximately 1 ^{o}C since the industrial age and is projected to rise by 2.6 to 4.6 ^{o}C by 2100 (IPCC, 2021). Climate change also results in changes in precipitation patterns and increasing climate variability (Tietjen et al. 2017; Huang et al. 2021). All these aspects related to climate change are anticipated to impact crop growth, yield, and food nutrient concentrations, consequently impacting human nutrition, particularly the nutrition of small-holder farmers (McMichael and Lindgren 2011). Further, as the global population is projected to grow by 25 percent in the next three decades, climate changes will pose severe challenges for crop production, exacerbating food insecurity and malnutrition (Hickey et al. 2019; Leisner 2020). Climate change, however, is yet to be thoroughly investigated in terms of its effect on fruit crop nutritional quality.

The impact of elevated carbon dioxide on the nutritional value of fruit and vegetable crops has been explored in some studies (Lieffering et al. 2004; Dong et al. 2018), while less is known about the impact of warming. Under elevated carbon dioxide, major crops, including wheat and potatoes, are more likely to increase yield (Ainsworth and Long 2005), but the nutrient accumulation in the edible portion of the crops decreases (Ebi and Loladze 2019). However, elevated CO_2 significantly increases phenolic compounds and flavonoid contents in strawberries (Wang et al. 2003). The impact of warming on the nutritional qualities of crops needs to be investigated. The production of some annual and perennial fruit crops, especially in

temperate regions have been noted to be affected by rising temperature (Sugiura and Yokozawa, 2004) and in water-limited regions (Parker et al. 2020). Warming will change the water transport and transpiration of plants, which will consequently change the nutrient uptake, transportation, and accumulation due to the coupling between water and nutrient assimilation (Zhang et al. 2018; Yin et al. 2022). In addition, warming can cause water and heat stress impairing plant physiology, carbohydrate metabolism, and sugar allocation to sink tissues, including the edible parts (Qi et al. 2022). Climate warming also influences the production and accumulation of antioxidant compounds such as anthocyanins and flavonoids, but the results are inconsistent among different fruit crops. For example, winter warming did not significantly affect the total anthocyanin content in blackcurrant (Pagter and Kjær 2022), while other studies noted a decrease in anthocyanin and flavonoid contents in grapes, red-fleshed kiwifruit, and strawberries (Barnuud et al. 2014; Man et al. 2015; Matsushita et al. 2016; Gouot et al. 2019).

Plant proteins are essential in photosynthesis and defense against environmental stressors (Rambal 1993; Rasheed et al. 2020). Leaf tissues and edible portions of plants store soluble proteins that meet the nutritional demands of plantlets (Day 1996; Rasheed et al. 2020) and inadvertently supply humans with essential amino acids (Millward et al. 2004). Global protein availability is predicted to decline by 2050 due to climate change (Myers et al. 2017; Ebi and Loladze 2019). A 23 percent reduction in the protein content of fruit crops under elevated CO_2 is observed, which will further exacerbate the damaging impact of climate change on major food crops (Medek et al. 2017). The climate change effects will be exacerbated in developing countries with a fast-growing population (IPCC, 2021). Total soluble solids (TSS) and titratable acidity (TA) are closely related to the ripening, flavor, and eating quality or acceptance of many fruit crops (Perkins-Veazie and Collins 2001; Mengist et al. 2020), while the ratio of TSS to TA

(ripening index) can be significantly affected by climate change (Milošević et al. 2012). The negative effect of climate change on TSS, TA, firmness, and texture can also result in a reduction in consumer acceptability (Sugiura et al. 2013). The recent decline in consumer acceptance of small fruit crops, may be due to climate change but has not been examined (Jaiswal 2020).

The wild blueberry (*Vaccinium angustifolium* Ait.) is a woody perennial crop native to North America. Maine produces the most significant commercial wild blueberries worldwide (Yarborough 1997), contributing more than $80 million in annual direct revenue (Yarborough et al. 2017). Wild blueberries have a good balance of soluble sugars, acids, and other primary metabolites and aromatic compounds, giving wild blueberries a range of good organoleptic properties (Antolín et al. 2020; Kalt and McDonald 1996). Wild blueberries have varied levels of citric, malic, quinic, and chlorogenic acids, leading to the development of value-added products like sparkling wine, juice, and snacks (Goueli 1976; Potter 2004). The mature wild blueberry fruits have a characteristic blue to dark purple coloration, which is attributable to the accumulated secondary metabolites (Brownmiller et al. 2008). When used as extracts in wild blueberry beverages and food products, these secondary metabolites promote gut microbiome and nutrition (Vendrame et al. 2011). In many studies, phenolic acids from wild blueberries have been used for pharmacological and clinical purposes, including antioxidant activity, cardiovascular disease risk management, and wound healing (Tsakiroglou et al. 2019). However, it has been documented that elevated surface temperatures have altered the metabolites composition and taste of other fruit crops such as apples and grapes (Yamada et al. 1994; Arrizabalaga et al. 2018).

The mean surface temperatures in Maine have increased by 0.6 ºC since 1959 (Fernandez et al., 2020), and wild blueberry farms have been warming more rapidly compared to the rest of

the region (Tasnim et al., 2021). Climate warming and drought alter the growing season length, physiological performance, growth, pest pressure, and berry production of wild blueberries (Barai et al. 2021; Chen et al. 2022). However, the response of wild blueberry nutritional quality to climate warming remains unknown.

To this end, this research aimed to:

1) Quantify the effect of warming on chemical compositions related to the nutritional quality of wild blueberries and

2) Test whether warming will change the associations among different metabolites.

I used controlled experiments in the field to study the response of berry sugars (glucose and fructose), total soluble solids, total soluble protein, anthocyanin, phenolic, microminerals, and macrominerals to two warming treatments, which increased atmospheric temperatures by 1.2 ^{o}C and 3.3 ^{o}C, respectively. Our study will contribute to the understanding of the effect of climate warming on the nutritional quality of perennial crops such as wild blueberries and provide needed scientific bases for the development of climate change mitigation strategies.

1.3 Materials and methods

1.3.1 Study Location

This research was conducted at Blueberry Hill Research Farm in Jonesboro, Maine (Longitude: 67.6495° N, Latitude: 44.6454° W) in 2019 and 2020. The mean annual temperature of the study site is 6.9 ^{o}C, and the mean annual precipitation is 1298 mm (NOAA, 2023). The wild blueberry experimental site comprises a well-drained Colton gravelly sandy loam with a soil pH of 4.7 (Smagula and Hepler 1978).

1.3.2 Experimental Design and Fruit Sampling

Open-top chambers were constructed for this experiment to simulate realistic warming scenarios (Tasnim et al. 2020). Briefly, both active and passive heating open-top chambers were designed with 55 cm high x 100 cm width dimensions slanted at 60 degrees on six sides to enclose wild blueberry plants using translucent polycarbonate sheets. The active heating chambers used heating tapes (Briskheat, Columbus, OH, USA) inside the chambers to continously raise the air temperature for two years (2019 and 2020), while the passive chamber had no heating tapes. The control condition has no open-top chamber (ambient). The average air temperature (Ta), relative humidity (RH), air vapor pressure deficit, and volumetric soil water content (VSWC) in 2019 and 2020 were recorded every 30 minutes by the Micro Stations (Spectrum Technologies Inc., Aurora, IL, USA) installed in the middle of the chambers, and raised to a height of approximately 10 cm above the soil. The active open-top chambers used in this study raised the ambient temperature by 3.3 °C on average, while the passive open-top raised the ambient temperature by 1.2 °C on average.

Five temperature treatment replicates were blocked by genotypes in a randomized block design. Trial plots were set up on six morphologically diverse genotypes in April 2019, and the warming treatments were applied for two years (2019 and 2020). Wild blueberry crops were managed as a two-year-cycle crop. The aboveground parts are pruned or burned after harvesting. Plants only have vegetative growth in the following year (vegetative growth year or prune year), and berry production begins in the subsequent year (crop year). Fruit quality samples were hand-picked only in mid-July to early August of crop year (2020) when approximately 90% of the berries are ripe and have dark blue colors. No fertilizer or irrigation were applied during the two years of study.

1.3.2.1 Determination of pH and Titratable Acidity (TA)

Frozen wild blueberry samples (5 g) were homogenized using a table-top waring laboratory blender (Waring Laboratory, Torrington, CT, USA) for 1 min. The blended wild blueberry puree from each sample was diluted with 50 mL of distilled water. The pH values were determined using a digital pH meter (Accumet® AB150, Fisher Scientific, Hampton, NH, USA). Before measurements, the pH meter was calibrated using standard buffers. Titratable acid (TA) was assessed by titrating 0.1 mol L^{-1} sodium hydroxide against diluted wild blueberry juice until a final pH of 8.2 was reached. Titratable acidity was expressed as percentage of citric acid concentration.

1.3.2.2 Determination of Total Soluble Solids (TSS)

Wild blueberries (100 g) were blended and filtered through a Millipore filter (0.45 um, Millipore Products division, Bedford, MA) to extract the juice. The filtered juice was used to determine the total soluble solids using a hybrid digital refractometer PAL-BXI ACID F5 (Atago Co. Ltd., Tokyo, Japan). Before TSS measurements, the digital refractometer was calibrated using distilled water, and the values were expressed in ^{o}Brix. The TSS: TA ratio was also calculated and compared among treatments.

1.3.2.3 Extraction and Quantification of Soluble Protein Concentration (SPC)

Five grams of frozen wild blueberries were pulverized for one minute with a table-top waring laboratory blender (Waring Laboratory, Torrington, CT, USA) containing 45 ml of freshly prepared phosphate buffer saline of pH 7.4. The homogenized solution was stirred in a beaker at 24 ^{o}C for 30 min. The stirred solutions were then centrifuged at 15000 rpm for 15 min. The soluble protein supernatants from each sample collected in a 50-mL falcon tube were quantified according to the Lowry method (Shen et al., 2013). The quantification was done by

diluting 100 uL of each duplicated sample to 500 uL with distilled water. Then 5 mL of freshly prepared reagent A (100 mL of 2% sodium carbonate in 0.4% sodium hydroxide + 1 mL of 1% copper sulfate + 1 mL of 2.7% sodium potassium tartrate) was added to the diluted samples, vortexed immediately before incubation for 10 min at room temperatures. Following this, 0.5 ml of freshly prepared reagent B (Folin–Ciocalteu phenol reagent in H2O (1:1 v/v)) was added to all tubes (in the same order as added in the previous step) and vortexed before incubation for 25 min at room temperatures. Samples were quantified at 700 nm absorbance using a spectrophotometer (BioTek Instruments, Winooski, VT, USA). A standard curve of 0, 0.0625, 0.1250, 0.2500, 0.5000, and 1 mg/mL concentrations was prepared with bovine serum albumin (BSA), from which soluble protein was determined. Wild blueberry soluble protein concentration was calculated as mg BSAE per 1 gram of fresh weight.

1.3.2.4 Sample Extraction for Total Phenolic, Flavonoid, and Anthocyanin Concentration

Five grams of pulverized frozen wild blueberry samples were weighed and placed in 25-mL glass beakers containing 10 mL of acidified methanol, followed by 10 min of sonication and filtration with a 0.45-μm filter. This process of sonication and filtration was repeated twice after the first time by adding 10 mL of acidified methanol to the filtrate (Nicoué et al. 2007). The final filtrate was then concentrated to almost dryness in a turbo valve evaporator under a 35 °C water bath with a vacuum and redissolved in distilled water to a final volume of 25 mL. The dissolved filtrate was stored at -20 °C for subsequent assays of total phenolic concentration (TPC), total flavonoid concentration (TFC), and total anthocyanin concentration (TAC).

1.3.2.5 Total Phenolic Concentration (TPC) Analysis

Colorimetric analysis using Folin-Ciocalteu reagents was used to quantify the total phenolic concentration in the wild blueberry extract as described by You et al. (2011) with slight modification. Briefly, 1 mL of thawed extracted samples was diluted with 9 mL of 100% methanol. After this, 20 uL of the diluted sample were transferred into a 2-mL Eppendorf tube containing 90 uL of diluted Folin-Ciocalteu with distilled water (1:10 H_20 (v/v) and held at ambient temperature for 5 min. Ninety microliters (90 uL) of 6% sodium bicarbonate solution were added, and the obtained mixture was vortexed thoroughly and incubated in the dark at room temperatures for 90 min. Samples were then transferred to a 96-well microplate reader (BioTek Instruments, Winooski, VT) to read the absorbance (750 nm) of the samples and the gallic acid standards (0-250 ug/mL). Each standard and the wild blueberry sample solution were analyzed in triplicate. The total phenolic concentration in the wild blueberry sample was expressed as micrograms of phenolic extract in gallic acid equivalents per gram of sample fresh weight (ug GAE/g FW) (Singleton et al. 1999).

1.3.2.6 Total Flavonoid Concentration (TFC) Analysis

The total flavonoid concentration (TFC) of the wild blueberry extracts was measured using the colorimetric method previously described by Chang et al. (2002) with slight modifications. Briefly, 0.6 mL of extract samples were mixed with 0.6 mL of 2% (w/v) $AlCl_3$ solution in methanol and vortexed. The same volume of 0.6 mL of the quercetin standards was mixed with 0.6 mL of 2% (w/v) $AlCl_3$ solution, then vortexed and kept at room temperature for 1 hr. The absorbance of the mixtures was read at 420 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA). Quercetin stock solution was prepared by dissolving 5.0 mg quercetin in 1.0 mL 100% methanol and then serially diluted (0-250 μg/mL) using methanol to

generate a linear calibration curve ($R^2 = 0.998$). The total flavonoid concentrations were expressed in milligrams per gram (mg QE/g) of the weight sample. Each standard and sample solution were measured in triplicate.

1.3.2.7 Total Anthocyanin Concentration (TAC) Analysis

With a UV-visible spectroscopy microplates reader (BioTek Instruments, Winooski, VT, USA), the absorbance of pH = 1.0 and pH = 4.5 buffer-diluted extract of wild blueberry total anthocyanin concentration were measured at 520 nm and 700 nm absorbance wavelengths following the pH differential methods described by (Lee et al. 2008). Measurement of samples was done in triplicate within 30 min of preparation. The monomeric anthocyanin concentration of each sample was calculated using the following equations:

1. *Total anthocyanins (mg/100g) = A= (Avis max−A700)$_{pH=1.0}$ − (Avis max−A700)$_{pH=4.5}$*

2. *Anthocyanins (mg/L)=(A×449.2×DF×1000)/ (26,900×1). Where A is the absorbance of the sample, and DF is the dilution factor.*

Anthocyanin concentration was expressed based on the molecular weight (449.2 g mol−1) and molar extinction coefficient (26,900 L cm−1 mol−1) of cyanidin-3-glucoside in an aqueous solution.

1.3.2.8 Soluble Sugar Extraction and Analysis

Wild blueberry sugar extraction was carried out following a previously described method by Zhang et al. (2020). Briefly, 10 g of homogenized wild blueberry fruits was suspended in 25 mL of 50:50 acetonitrile: distilled water in 50-mL polyethylene centrifuge tubes. A tissue homogenizer (polytron) was used to pulverize the tissue for 2 min to release the sugars. The samples were then centrifuged at 7,000 x g for 10 min. An aliquot of about 1-2 mL of the sample

supernatant containing soluble sugars was filtered through a Millipore filter (0.45 um, Millipore Products division, Bedford, MA) fastened to a sterile syringe nylon disc and transferred to HPLC sample vials. The sugar determination was performed using high-performance liquid chromatography (HPLC) following a previously described method (Zhang et al. 2020). The results were expressed as mg/g fresh weight (FW).

1.3.2.9 Macro and Microminerals Determination

Oven-dried and ground wild blueberries were sent to the Plant and Soil Testing Laboratory, University of Maine, Orono, to analyze macronutrient and micronutrient concentrations. Non-volatile nutrient concentrations in wild blueberries were determined by ashing 50 g of fruit tissue samples at 550 °C for 5 hr in a muffle furnace. Briefly, total digestion was conducted following the EPA 3050B method described by Kaltra and Maynard (1991), in which a cooled ashed sample was dissolved in 50 % HCl on a hotplate and brought to volume with Deionized water. Wild blueberry fruit was digested and analyzed for N, Ca, K, Mg, Al, Bo, Cu, Fe, Zn, P, and Mn using coupled plasma optic emission spectrometer (ICP-OES; Thermo-Jarrell Ash model IRIS 1000 dual-view, Franklin, MA, USA). Samples were extracted and digested in triplicate, along with standard reference material, to ascertain the accuracy of the procedure.

1.3.3 Statistical Analysis

Statistical analyses were performed in the R core environment (R Team). Each macro and micronutrient were calculated as the mean of three replicates + standard error (SE). All the quantified values (pH, %TA, TSS, SPC, TPC, TFC, and TAC) were checked for normality and used raw for analysis except for the total anthocyanin, soluble sugars, and iron values, which were log-transformed. The phosphorus macroelement failed the normality test even after log

transformation, so a non-parametric Kruskal-Wallis's test was conducted. The total soluble solids to titratable acidity ratio were estimated by dividing the °Brix value by % TA. A lmer function in R was used to develop a linear model in which clones were treated randomly, and treatment factors (control, passive heating, and active heating OTCs) were fixed (De Boeck et al., 2011). Significant differences were completed using two-way or multifactorial ANOVA with a significant test evaluated at $p \leq 0.05$. For post hoc analyses, Fisher's Protected Least Significant Difference (LSD) test was used to analyze the significant mean separation between all treatments in R studio (R Team; Allaire 2012).

1.4 Results

1.4.1 Effect of Warming Treatments on Wild Blueberries Chemical Composition

While noticeable changes in berry chemical composition were found in both climate warming treatments (passive heating and active heating) when compared to the ambient temperature, active heating treatment exerted the most measurable effect on berry chemical composition (Figure 1.1 & 1.2). The active warming treatment caused a significant reduction ($p < 0.05$) of 13.4 percent in the total soluble solid's concentration compared to the ambient control (Figure. 1.1c). However, warming treatments showed no significant effect on berry pH and titratable acidity compared to the ambient control (Figure 1.1a, b).

The total soluble solids (TSS) to the titratable acid (%TA) ratio is a good index to indicate berry ripening. The TSS to %TA ratio showed no significant difference among treatments (Figure 1.1d).

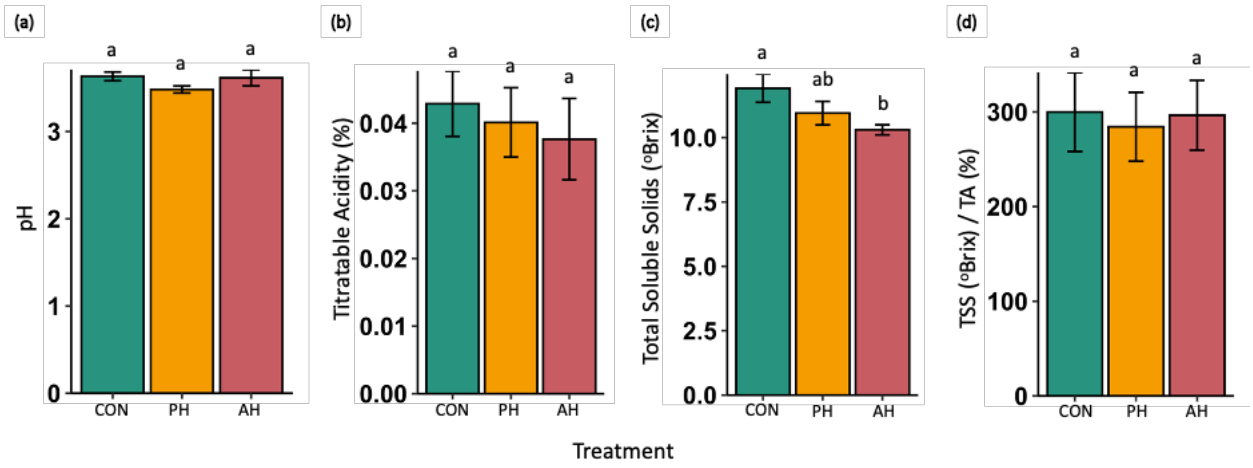


Figure 1.1: The pH (a), titratable acid (b), total soluble solids levels (TSS) (c), and the ripening index (the ratio of total soluble solids to titratable acidity) (d) in wild blueberry fruit under different temperature treatments. CON: control ambient; PH: Passive heating treatment; AH: Active heating treatment. Bars are means + SEs (n = 6). Bars having the same letter were not statistically different ($p > 0.05$).

1.4.2 Effect of Warming on Wild Blueberries Soluble Sugar Concentrations

High-performance liquid chromatography analysis of wild blueberries under our experimental conditions did not detect sucrose at a limit of 0.1 mg/g. Thus, our analyses focused on concentrations of detected glucose and fructose. Wild blueberries under the active warming treatment had the lowest fructose concentrations, which is significantly lower than for ambient control (Figure 1.2a). Warming treatments did not differ significantly from ambient control in glucose concentration (Figure 1.2b). Total soluble sugars of wild blueberries under active warming treatment showed significant reduction ($p < 0.05$) compared to the ambient control (Figure 1.2c).

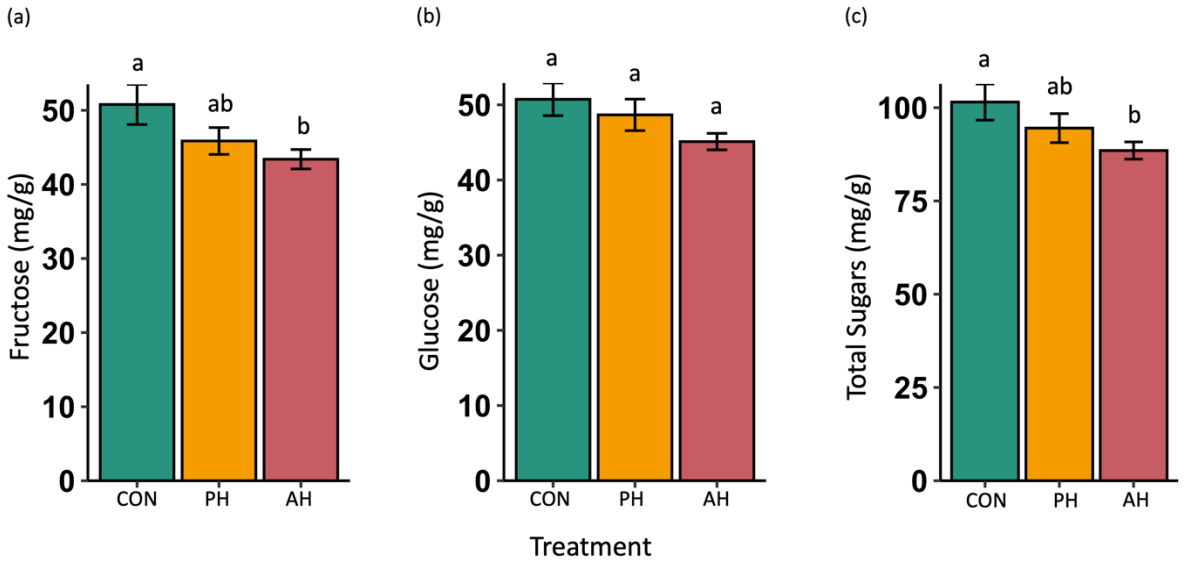


Figure 1.2: The soluble sugar concentration of wild blueberries under different temperature treatments. The mg/g FW of fructose (a) and glucose (b) and total sugar (c) from wild blueberry fruits under different treatments: CON (controls), PH (passive heating), and AH (active heating) are presented. Bars represent means and standard errors of at least five replicated clones. Clones are treated as random effects, while treatments as fixed effects. There is no significant difference in bars that have the same letter ($p > 0.05$).

1.4.3 Effect of Warming on Wild Blueberries TAC, TPC, TFC, and SPC

No significant differences in total anthocyanin, flavonoid, and phenolic concentrations were found in wild blueberry fruits under different treatments (Figure 1.3a to 1.3c). The total soluble protein concentration in wild blueberries showed significantly and distinctly lower ($p < 0.05$) concentrations in warming treatments (PH and AH) than for ambient control. Under the passive treatment, the total soluble protein concentration (0.68 ± 0.07) was reduced by 25-30% compared to the ambient control (1.033 ± 0.07). Under the active treatment, total soluble protein (0.508 ± 0.08) was reduced by 50% in berries compared to the control (Figure 1.3d).

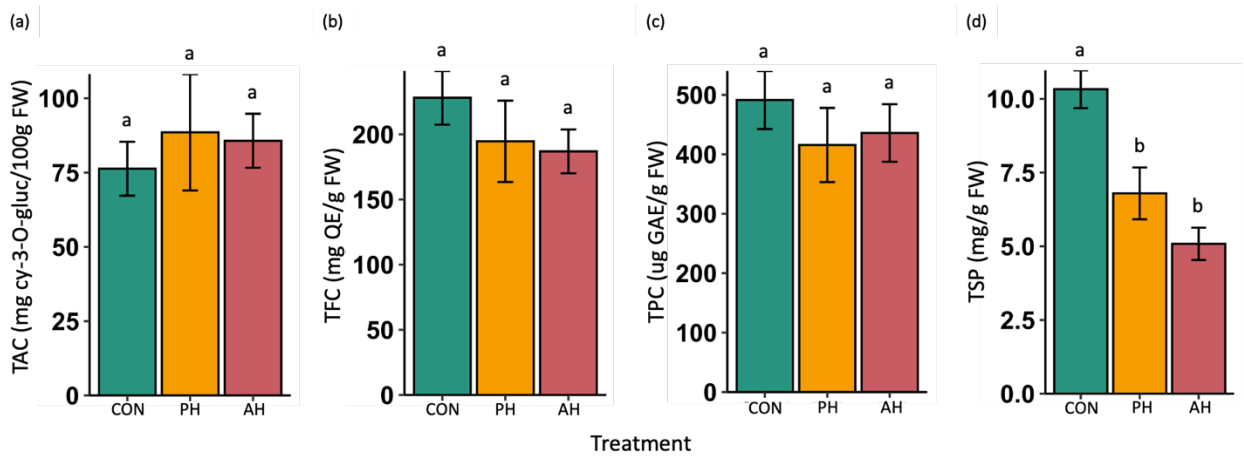


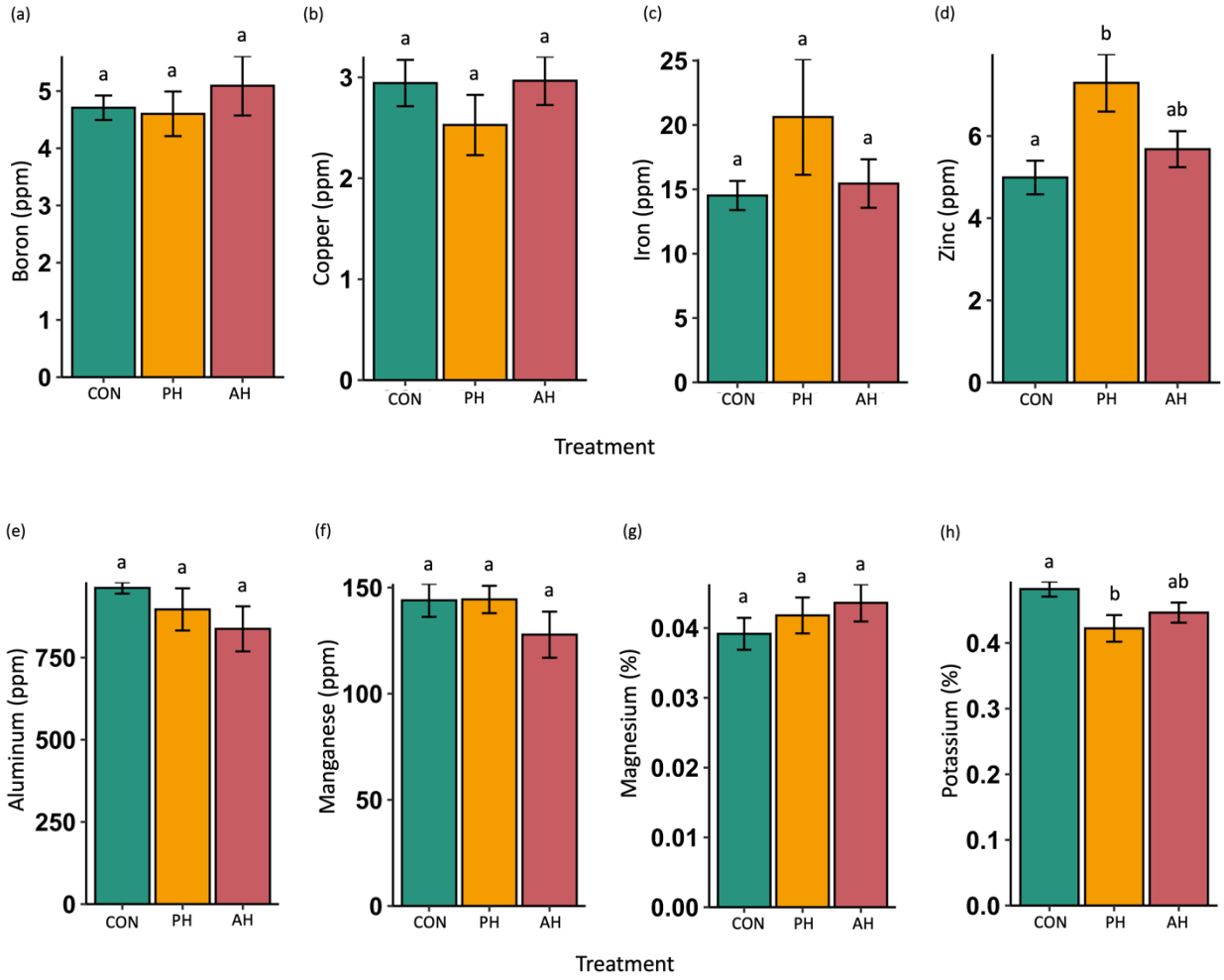
Figure 1.3: Total anthocyanin (a), total flavonoid (b), total phenolic (c), and total soluble protein (SPC; d) concentration from fresh-weight wild blueberries under different temperature treatments. Three treatments: ambient control (CON), passive heating (PH), and active heating (AH). Total flavonoid concentration was not significantly different under warming treatments (b), while the total soluble protein was significantly different (d). Error bars indicate standard errors. Different letters are significant at an alpha level of ($p < 0.05$).

1.4.4 Effect of Warming on Wild Blueberries Micro and Macrominerals

No significant impact of warming temperatures was observed in wild blueberry fruit concentrations of boron, copper, iron, aluminum, and manganese (Figure 1.4). Wild blueberry plants under passive warming treatment showed significantly higher ($p < 0.05$) zinc concentrations compared to plants in the ambient control (Figure 1.4d). The zinc concentration was 25-30% higher in fruits of wild blueberry under passive treatment than in control. However, the zinc concentration was not significantly different in wild blueberries under active treatment compared to control.

Regarding the macro minerals, wild blueberry magnesium and nitrogen concentrations were not significantly different among different treatments (Figure 1.4 g,j). The calcium concentration in wild blueberry fruits was significantly higher ($p < 0.05$) under the passive heating treatment, while the phosphorus concentration of wild blueberry fruits was significantly increased ($p < 0.05$) under the active heating treatment compared to the ambient control (Figure

1.4 i, k). Wild blueberry potassium concentration was significantly lowered ($p < 0.05$) in berries under the passive warming treatment by 20% compared to the control (Figure 1.4 h).



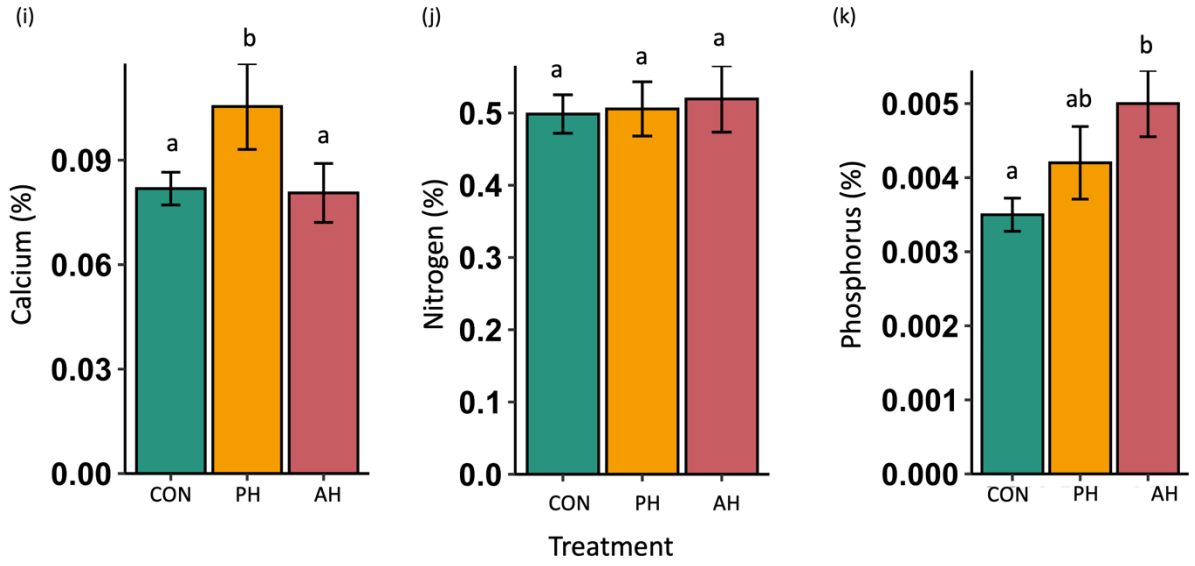


Figure 1.4: The average concentration of essential microminerals and macrominerals in mature wild blueberries under different treatments. Boron (a), copper (b), iron (c), zinc (d), aluminum (e), manganese (f), magnesium (g), potassium (h), calcium (i), nitrogen (j) and phosphorus (k) concentration under different warming treatments: ambient control (CON), passive heating (PH), and active heating (AH) treatments. Error bars are standard errors of the mean. Bars with different letters depict a significant difference ($p < 0.05$) among different warming treatments.

1.4.5 Correlation between Wild Blueberries Composition Effect

To show the strength and the direction of the relationship between chemical measurements, we conducted a correlation analysis on wild blueberry fruit variables for all treatments (Figure 1.5). As expected, under ambient conditions sugars (both glucose and fructose) showed strong positive and significant correlations with total soluble solids ($p < 0.05$), while total flavonoids, phenolic, and anthocyanin contents were weakly correlated to total soluble sugars (Figure 1.5a). Notably, titratable acidity is negatively and poorly correlated with total soluble solids and positively correlated with total soluble proteins under ambient condition (Figure 1.5a). However, this relationship of TA to TSS and TSP changed under passive and active warming conditions (Figure 1.5b & c). The ripening index and flavor indicated by the TSS/TA ratio showed a positive relationship with TPC, TFC, and TAC under the ambient and

warming treatments (Figure 1.5a, b & c). Furthermore, when comparing the relationship between total soluble protein (TSP) and secondary metabolites (TAC, TPC and TFC), under ambient temperature, and warming treatments, we found that the TSP negatively correlated with TAC, TPC, and TFC under ambient conditions, while the relationship changed to positive under elevated surface temperatures (Figure 1.5a, b & c).

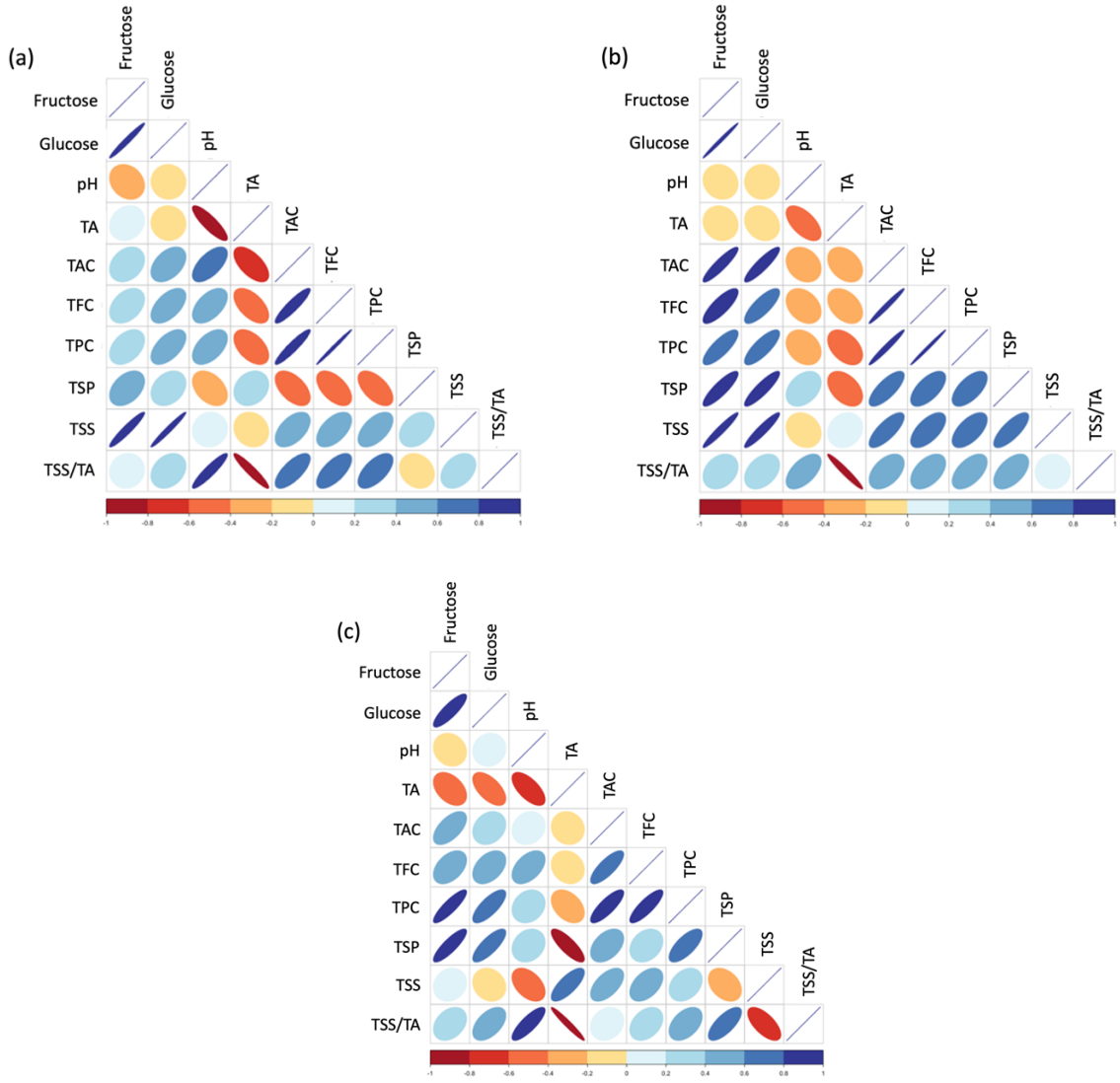


Figure 1.5: Pearson correlation matrix of wild blueberry nutritional attributes (TPC, TFC, TAC, SPC, TSS, pH, TA, Fructose, Glucose) under (a) ambient temperatures, (b) passive warming, and (c) active warming treatments. The magnitude of correlations is represented by the shape of the cloud, while the direction is represented by the colors: blue (positive correlation) and red (negative correlation) between measurements.

The predictive ability of sugars from total soluble solids (measured with widely available refractometer like Brix meter) within different conditions was tested. There was a positive and significant linear relationship between glucose concentration and total soluble solids for the ambient control ($p < 0.001$) (Figure 1.6). Similarly, we also found a significant and positive relationship under the passive heating treatment among plots under the passive heating treatment ($p < 0.05$). However, the relationship between glucose and total soluble solids in the active heating treatment was insignificant (Figure 1.6a). A positive and significant linear association was found between fructose concentration and total soluble solids for the ambient conditions and passive heating treatments but not the active warming treatment (Figure 1.6a).

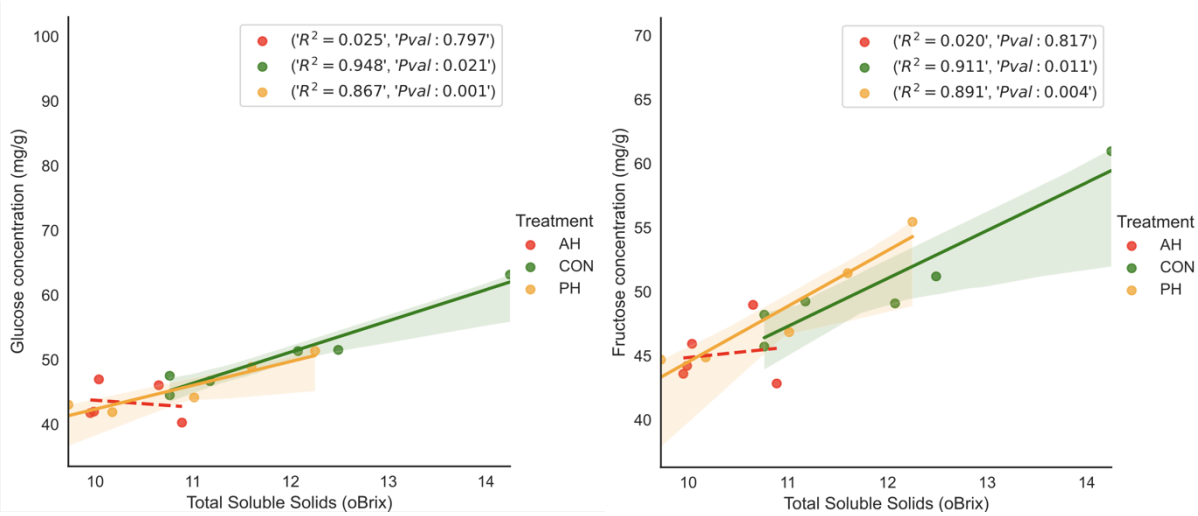


Figure 1.6: Linear association of glucose concentration with total soluble solids (a), and fructose concentration to total soluble solids (b). Green, orange, and red line and dots indicate the ambient control, passive heating, and active heating treatments, respectively. The lines indicate linear relationships for corresponding treatments: control, passive, and active heating treatments. The plot shows R^2 and p values for different treatments are shown in the upper right box.

1.5 Discussion

Our results showed that warming by 1.2 to 3.3 °C during the cropping cycle (two years) significantly reduced the total soluble solids, fructose concentration, total soluble sugars, and

total soluble protein concentration of wild blueberries. Nutrients such as phosphorus, zinc, and calcium increased while potassium concentration was reduced in wild blueberries under elevated temperatures. Temperatures are predicted to rise by 2.6 to 4.6 ºC by 2100 (IPCC, 2021). Our studies thus highlight the changes imposed by warming in this century on wild blueberry nutritional quality, which may impact consumer preferences.

Effect of Warming on TSS, Soluble Sugars, TA, and pH

We found that warming decreased total soluble solids (TSS) concentration by 8.4 percent (passive heating) and 13.4 percent (active heating) of wild blueberries, respectively, compared to the ambient control. TSS, which comprises soluble sugars, amino acids, and other dissolved organic substances, is a vital indicator of the sweetness of wild blueberries (Kusumiyati et al. 2020). The average TSS of mature wild blueberries grown under ambient conditions in this study is 11.9 percent, which agrees closely with the observed mean TSS contents of ripe wild blueberries (Kalt and McDonald 1996). The declines in TSS under warming treatments agree with previous studies for other crops such as Kiwifruits, beetroots, and strawberries (Hopkirk et al. 1989; Juszczak et al. 2010; MacKenzie et al. 2011; Menzel 2022). Higher temperatures can result in higher cellular respiration and quicker fruit development or maturation, reducing the resources and time for the synthesis of TSS (Lopresti et al. 2014; Menzel 2022). Warming can also decrease enzyme kinetics involved in berry sugar synthesis (Bharti et al. 2021; Lv et al. 2021). Additionally, warming can decrease soil moisture content, increase plant respiration, and enhance water stress (Parmesan and Yohe 2003; Myers et al. 2017; Menzel 2022), which may all contribute to reduced soluble solids. For instance, high temperatures induced water stress causing a reduction in TSS in peaches (Besset et al. 2001).

A decline in fructose in wild blueberries under elevated temperatures, as found in this study, was also found in strawberries (Wang and Camp, 2000). Soluble sugar concentration is a vital fruit trait for wild blueberries as it strongly determines the taste. Wild blueberries taste sweet due to their relatively high sugar concentration, which is over 95% of their total soluble solids (Kalt and McDonald 1996). Enhanced water stress due to warming and increased water loss could result in sugar remobilization to belowground tissues, thus disrupting the accumulation of soluble sugars in berries (Zheng et al. 2020; Kim et al. 2021). Since soluble sugars contribute significantly to the sweet taste, future climate change may impact the preferences of berry consumers. Consequently, this can potentially affect global diets and the socioeconomic livelihood of wild blueberry growers (Giulia et al., 2020).

Titratable acidity measures the total organic acid content, another vital nutritional quality trait of fruit crops. Rising average pre-harvest and average temperatures have reduced titratable acidity in blackberries (Naumann and Wittenburg 1980). Fortunately, we found that warming did not significantly affect total titratable acids in wild blueberries. Titratable acid levels were consistently lower in blueberries of larger sizes (Uhe, 1957). However, despite the increased wild blueberry size observed under warming conditions in a previous study (Chen et al. 2022), a reduced titratable acidity was not found in this study.

Consumers enjoy incredibly flavorful wild blueberries, thus making flavor an essential nutritional quality trait. Titratable acidity (TA) and TSS concentration are indicators of the flavor and maturity of berries (Gilbert et al., 2015). Our data did not find any significant changes in the TSS/TA ratio and pH under the warming scenarios indicating that the ripening and flavor of the berries will not be affected. There are mixed previous reports supporting the influence of elevated temperatures on the TSS/TA ratio and pH. For example, TSS/TA ratio in apricots and

blackcurrant was lowered in seasons experiencing high temperatures (Bartolini et al. 2015; Pagter and Kjær 2022). However, the sea buckthorn TSS/TA ratio was reported to be higher at lower latitudes (Zheng et al. 2012).

Declined Total Soluble Protein under Warming

Our data revealed that total soluble protein in wild blueberries decreased significantly under the passive and active warming chambers compared to the ambient temperatures. The total soluble protein was reduced by half when temperatures were elevated by 3.3 ^{o}C, indicating the sensitivity of plant proteins to climate change. Like our findings, strawberry plants showed a lowered concentration of total soluble proteins under higher temperatures (Ledesma et al. 2004). Fruit crops exposed to severe heat stress, may have their defense proteins (HSPs and antioxidant proteins) inactivated or degraded (Ergin et al., 2016). Soluble protein is sensitive to abiotic stress associated with climate change, as a similar impact was observed under elevated CO_2 (Ebi and Loladze 2019). Under elevated temperatures, free amino acids increase due to the inevitable catabolism in storage soluble protein concentration in hard fescue cultivars (Wang et al. 2018), which may be a similar process occurring in the edible portion of the wild blueberry plants.

Effect of Warming on TAC, TPC, and TFC

Previous studies revealed that elevated temperatures reduce anthocyanin and flavonoid contents in major fruit crops such as grapes, red-fleshed kiwifruit, and strawberries (Barnuud et al. 2014; Gouot et al. 2019; Man et al. 2015; Matsushita et al. 2016). However, in our study, elevated temperatures (3.3 ^{o}C higher than the ambient temperatures) did not cause any significant change in the total anthocyanin, phenolic, and flavonoid concentrations in wild blueberries. Similar to our studies, mild winter warming did not cause any significant impact on the total anthocyanin content in blackcurrants (Pagter and Kjær 2022). In addition, the elevated

temperatures by 3.3 ºC might not disrupt the activity of flavonoid synthesis, thus causing no significant impact on the flavonoid and phenolic content of wild blueberries. On the other hand, warming-induced drought caused an increase in the anthocyanin contents in jujube fruits (Jiang et al. 2020b).

Effect of Warming on Macro and Microminerals of wild blueberries

Elevated temperatures (either passive or active treatments) decreased berry potassium and increased calcium, phosphorus, and zinc. However, it did not affect magnesium, nitrogen, boron, copper, aluminum, iron, and manganese concentrations. Elevated surface temperatures did not significantly lower the manganese concentrations in wild blueberries, thus remaining a good source of manganese (Bushway et al. 1983). Wild blueberries are perennial crops that grow in sandy soils and may be susceptible to climate change due to increased soil water deficits. The lack of coordinated soil water transport could affect minerals and ion movement in the soil (Qi et al. 2022). The relationship may be drastic under high temperatures, and that impact remains a significant concern for food quality and systems (Owino et al. 2022). Elevated temperatures promote evapotranspiration causing high water deficits in sandy soils, which, based on our study, decreased the wild blueberry potassium nutrients uptake, increased wild blueberry calcium, phosphorus, and zinc concentration, while wild blueberry magnesium, nitrogen, boron, copper, aluminum, iron, and manganese concentrations were unaffected. The inconsistent effect of elevated surface temperatures on nutrients is similar to that of zinc and iron concentration in rice grown under elevated CO_2 (Lieffering et al. 2004; Zhu et al. 2018).

Relationships among Wild Blueberries Quality Traits

In this study, the TSS/TA ratio, an index of ripening, maturity, and flavor for many fruit crops (Milošević et al. 2012; Mengist et al. 2020), showed a stronger positive relationship with

TPC, TFC, and TAC under ambient temperatures than under elevated temperatures. This may indicate a disruption in the relationship between ripening index and these secondary metabolites under high surface temperatures. Our findings agree with previous studies reported that elevated temperatures decouple the association between primary and secondary metabolites (Arrizabalaga et al. 2018). The TAC had a strong and positive correlation with TPC and TFC under ambient, and the relationship between them was still maintained under elevated temperatures. A previous experiment showed similar results, suggesting that the antioxidation and health-promoting properties were still maintained under high temperatures (Reyes-Carmona et al. 2005).

TSS measured by portable meters could be used as a quick indicator of soluble sugar concentrations in the field or when HPLC and LC-MS are not accessible. However, our study suggests that warming will alter the association of TSS with soluble sugars (Figure 1.6a and 1.6b). Similarly, the strong positive relationship between TSS and soluble sugars under ambient conditions became weaker or absent under warming (Figure 1.5). These results may be limited by the size of the samples, so in future study, the linear relationship between TSS and soluble sugars under active heating should be further investigated with larger sample size.

1.6 Conclusion

This research is the first to report the impacts of climate warming on wild blueberry nutritional quality and values. We found a significant reduction of fructose, total soluble solids, total soluble sugars, and total soluble protein under simulated climate warming conditions. All of these can reduce sweetness of wild blueberries, impacting the taste and content of wild blueberries. A consumer acceptance study of wild blueberries under climate change scenerios is warranted. Further experiments should compare the combined effect of elevated CO_2, high temperatures, and drought on the wild blueberry nutritional values and taste. Since the downeast

region fields were found to be warming faster than other regions in Maine (Tasnim et al. 2021), efforts to improve the water holding capacity such as mulching, and biochar should be investigated. Water and fertilizer management may need to be modified to maintain the high nutritional and health-promoting values of wild blueberries.

CHAPTER 2

CAN SOFTWOOD AND BIOCHAR MULCHES MITIGATE THE NEGATIVE IMPACT OF WARMING ON THE NUTRITIONAL QUALITY OF WILD BLUEBERRIES?

2.1 Abstract

Climate change affects not only the yield but also the nutritional qualities of crops. Perennial crops are particularly vulnerable to climate change and thus require mitigation techniques. To mitigate the adverse effect of warming on soil water and nutrient availability, sustainable and cost-effective soil management practices such as sawdust mulching and biochar-compost mix, could be used. Thus, we assessed the potential of using a biochar-compost mix and mulching to mitigate the negative impacts of climate warming on the nutritional quality of wild blueberries (*Vaccinium angustifolium* Ait.). Six wild blueberry genotypes were selected, and each genotype was treated with ambient conditions, ambient conditions with a biochar-compost mix, and three warming treatments (warming only, warming with softwood bark mulching, and warming with biochar-compost mix). We confirmed that warming negatively affected berry nutritional quality (total soluble solids, soluble protein, and secondary metabolites). Biochar-compost mix, and mulching did not reduce the negative effect of warming on wild blueberry fruit quality except for potassium and magnesium mineral concentrations. Additionally, berry size was positively related to water content but negatively with total soluble solids, organic acids, and secondary metabolites. These results suggest that the biochar-compost mix and mulching, at least the rate we tested, could not mitigate the negative impact of warming on berry nutrition. Higher rates of biochar-compost mix and mulching should be investigated for potential mitigation benefits.

2.2 Introduction

Climate change has become a pertinent challenge in agricultural production worldwide (Adams et al. 1990; O'Brien and Leichenko 2000; Rosenzweig et al. 2014). Unprecedentedly elevated temperatures have been reported to change the crop growth pattern, flowering time, emergence of pollinators, pest pressure, crop yield, and nutritional qualities (Gunathilaka et al. 2017; Chen et al. 2022; Alaba et al., unpublished). Climate warming also increases crop water use and evapotranspiration, enhancing soil water deficits and exacerbating drought effects in dry years (van Asten et al. 2011; Barai et al. 2021; Tasnim et al. 2021). Various adverse effects of climate change have been documented on major food crops such as rice, wheat, and corn (Ali et al. 2017). Climate change, both warming and elevated CO_2 concentration, can alter nutrient assimilation and crop quality (Medek et al. 2017; Myers et al. 2014), bringing uncertainties on sufficient nutrient supply to human beings. As greenhouse gas emissions and population continue to increase, sufficient and sustainable food production will be challenging unless mitigation techniques are implemented (Hall et al. 2017; Leisner 2020; Wheeler and von Braun 2013).

Soil amendments such as mulching, and biochar applications can be used to mitigate the enhanced water and nutrient deficits under warming conditions. Mulching fields with wood-based products such as paper and sawdust, is a sustainable and cost-effective agricultural practice that significantly benefits crop production by lowering weed spread, reducing soil compaction, and increasing soil mineralization (Chen et al. 2007; Haapala et al. 2014; Iqbal et al. 2020; El-Beltagi et al. 2022; Gumbrewicz and Calderwood, 2022). Moreover, pine bark mulch has been shown to mitigate water stress in wild blueberry fields, where warming-induced droughts affect crop productivity (Hunt et al. 2010; Barai et al. 2021). Biochar and biochar-compost mix

application are other soil amendment techniques that have been shown to increase soil water and nutrient holding capacity in farms (Schmidt et al. 2014; Sorrenti 2015). Wood-based biochar is produced through pyrolysis and has been demonstrated to mitigate the negative effect of climate change, such as drought (Shang 2019; Kalu et al. 2021). Biochar application across whole fields has been suggested as an efficient and cost-effective approach for sustainable crop production and improving overall soil health (Pratt and Moran 2010; Vijay et al. 2021). The wood-based biochar application has been reported to increase some biochemical properties of food crops. Minerals, such as potassium and magnesium, were absorbed and accumulated in peas and barley storage tissues after biochar was applied in soils (Kalu et al. 2021; Sorrenti 2015). Moreover, soil amendments with biochar increased total phenols and antioxidant activities in tomato fruits (Petruccelli et al. 2015). However, biochar application on boreal and temperate soils showed a minimal positive effect on the yield and production of economically important food crops such as barley, maize, and wheat (Kalu et al. 2021; Karer et al. 2013). Whether mulch and wood-based biochar applications can mitigate the negative impact of elevated temperatures on the nutritional qualities of perennial crops is unknown.

Wild lowbush blueberry (mainly *Vacinnum augustifolium*; Ericaceae) is an important native fruit crop in North America but is now under the threat of climate change. Wild blueberry pulps have a sweet taste, and their dark blue skins are rich in phenolic compounds and anthocyanin, which provide consumers with nutritional and health-promoting antioxidants (Kang et al. 2015; Kalt et al. 2000). The state of Maine, the largest commercial producer of wild blueberry, has been experiencing unprecedented climate warming and frequent drought in recent years (Barai et al. 2021; Tasnim et al. 2021). The drought impact and enhanced crop water use under warming could be mitigated by irrigation, but only ~30% of the wild blueberry fields are

covered with irrigation (Yarborough 2004). Irrigation systems are limited by water access and are not affordable for small growers. Climate warming can reduce their nutritional qualities of wild blueberries. A recent study found that elevated air temperature altered the nutritional qualities, which may impact consumers preferences of berries (Alaba et al., unpublished). Therefore, there is a need to test whether soil amendment approaches such as mulching, and the application of biochar-compost mix can mitigate the impact of climate warming on the nutritional quality of wild blueberries under field conditions.

This study aimed to examine the impact of mulching and biochar-compost mix application on wild blueberry fruit quality under climate-warming conditions. As the wild blueberry fields are mainly characterized as gravelly sandy loam soils with low water and nutrient holding capacity, mulching and biochar-compost mix application can potentially improve the water and nutrient conditions of wild blueberry fields. Therefore, we evaluated the effectiveness of biochar-compost mix and mulching application in mitigating the detrimental impact of climate warming on the quality attributes of wild blueberries by comparing them to unamended ambient control plots. We hypothesized that warming treatment would negatively impact the nutritional quality of wild blueberries, while mulching and biochar-compost mix application in wild blueberry fields would mitigate the impact, especially because of mulching and biochar-compost mix influence on soil water and nutrient retention.

2.3 Materials and Methods

2.3.1 Experimental Field Sites And Design

This experiment was conducted at the Blueberry Hill Research Farm (44.6° N, 67.6° W) in Jonesboro and Wyman's Farm (44.7° N, 68.0° W) in Deblois, Washington County, on the easternmost coast of Maine, at an elevation of 125 m. The average temperature and precipitation

were 8.1 ºC and 1152.65 mm in 2021 and 7.7 ºC and 1352.29 mm in 2022, respectively (NOAA, 2023). Hexagonal chambers were built using a transparent polycarbonate sheet with dimensions: 100 cm (base length) x 70 cm (top length) x 55 cm (height). In the chambers, heating tape (Briskheat, Columbus, OH, USA) systems were installed to consistently raise the ambient temperature by 3-5 ºC. Treatments were applied for two years for two years (2021 and 2022), with no irrigation or fertilizer application during the experimental study. Each field site was set up with three morphologically diverse wild blueberry genotypes. Each genotype contains two plots under ambient conditions with biochar-compost mix (CON_BCM) and without biochar-compost mix (CON) and three warming open-top chamber plots without biochar-compost mix (AH), with soil topped with softwood bark mulch (AH_M), and biochar-compost mix (AH_BCM) respectively. Overall, five treatment replicates were blocked by genotypes in a randomized block design. Softwood bark mulch was spread across the soil surface and had a thickness of approximately 1.3 cm. The biochar and compost were mixed (in a ratio of 1:1) and uniformly spread at a rate of 7.5 cubic yards per Area (yd^3/A). Microclimatic conditions, including atmospheric temperature and relative humidity, were monitored, and recorded every 30 minutes in each block between June and November 2021 using HOBO weather stations (Onset Computer Corp., Bourne, MA) installed in the middle of the chambers, and raised to a height of approximately 10 cm above the soil. Sampling for fruit quality assessment were carried out in July 20, 2022 by hand-picking when approximately 90% of the berries are ripe and have dark blue colors in treatment plots.

2.3.2 Berry Physicochemical Analyses

The fresh weight (FW) of ripe berry samples (100 g) and dry weight (DW, g), following drying at 70 ºC for 36 hr were measured. Twenty fresh berries from each treatment were

randomly selected, weighted individually, and for subsequent berry diameter (mm) measurements using image J software (Schindelin et al. 2012). Wild blueberry samples (5 g) were macerated in 60 mL of deionized water using a waring blender (Waring Laboratory, Torrington, CT, USA) for 1 min. The filtered juice was used to assess pH using a pH meter (Accumet® AB150, Fisher Scientific, Hampton, NH, USA), and titratable acidity (TA), measured after titrating at pH 8.2 with 0.1N NaOH and expressed as a percentage of citric acid; total soluble solids (TSS), assessed using a refractometer (Atago, PAL-Plato), expressed as ^{o}Brix; and ripening index (RI), calculated by the ratio of TSS and TA. The remaining berries were immediately placed in -80^{o}C storage for subsequent use.

2.3.3 Berry Moisture Content

Wild blueberry moisture content was determined by measuring 100 g of the sample after 48 h of drying at 70°C according to Canet (1988). The berry moisture content was expressed as the percentage of fresh weight.

2.3.4 Chemical Analyses

2.3.4.1 Organic Acid and Soluble Sugars Extraction and Analysis

Whole wild blueberry samples (200 g) were blended using a Robot Coupe R401B food processor (Ridgeland, MS). 5 g of blended samples was homogenized using Polytron® tissue homogenizer for 1 min in 25 mL of 10 mM K_2HPO_4 (pH adjusted to 2.6 with H_3PO_4) for organic acids extraction and in 25 mL of acetonitrile: water (50:50 (v/v)) for soluble sugars extraction. The extracts were then centrifuged at 7,800 rpm for 10 min. From the supernatants, 1 mL aliquot was transferred to an HPLC sample vial for each assay.

High-performance liquid chromatography (HPLC) analysis was conducted to identify and quantify the organic acids using an Agilent 1100 series HPLC system with Chemstation® software. The analytical columns used were the Restek Allure Organic Acids® (300 mm x 4.6 mm I.D., 5 μm) for organic acids and Shodex NH2P-40 3E (250 mm x 4.6 mm I.D., 3 μm) for soluble sugars. The organic acid column was held at ambient temperature, while the sugar column was held at 35˚C. The mobile phases were 10 mM K_2HPO_4 at a pH of 2.6 for organic acids and acetonitrile: water (65:35 (v/v)) for soluble sugars. For organic acids, the separation was performed at a flow rate of 0.80 ml min^{-1} with a one-microliter injection volume. The diode array detector (DAD) on the Agilent 1100 series HPLC system was set to monitor signals at 210 and 226 nm. The soluble sugars were separated at a flow rate of 0.40 ml min^{-1} with a 5-microliter injection volume. The refractive index detector on the Agilent 1100 series HPLC system was set to the positive mode and maintained 35˚C. Analytical standards were prepared and used to identify and quantify target analytes for both HPLC methods. Retention times and areas of the peaks generated from analytical standards were used to identify and quantify peaks from samples. Peak areas were calculated by manual integration from chromatograms generated by Agilent Chemstation® software.

2.3.4.2 Secondary Metabolites and Antioxidant Analysis

2.3.4.2.1 Total Anthocyanin Content (TAC) And Total Phenolic Content (TPC) Assay

Frozen blended samples (5 g) were homogenized with 10 mL of methanol: formic acid extraction solution (99.9: 0.1, v/v) using a Polytron® tissue homogenizer (Kinematica, Switzerland) for 1 min. The mixtures were sonicated thrice at an interval of 10 min and then centrifuged at 7,000 x g for 5 min. Each time between sonication and centrifugation, the supernatant was transferred into a new 50-mL Falcon tube, and 10 mL of methanol: formic acid

extraction solution was added. The final supernatant was filtered through a Whatman® 0.45 µm folded filter paper (Thermo Fisher Scientific, Waltham, MA) and then dried using a Turbo-Vac II nitrogen evaporator (Zymar, Hopkinton, MA). After completely drying the extract, 25 mL of deionized water was added, and the sample was stored at - 20 ºC before analyzing total anthocyanin content (TAC), total phenolic content (TPC), and ferric-reducing antioxidant power (FRAP). TAC was measured in triplicate using pH differential methods described by Lee et al. (2008)**.** TPC was quantified in triplicate using the Folin-Ciocalteu reagent described by You et al. (2011).

2.3.4.2.2 Ferric-Reducing Antioxidant Power (FRAP) Assay

FRAP assay was performed following the method described by Benzie and Strain (1996). Briefly, fresh FRAP reagent was prepared by stirring 300 mM sodium acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ), and 20 mM FeCl3.6H2O in the ratio (10:1:1 (v/v/v)). For the determination, 3 mL FRAP reagent was added to 100 uL of the sample extract and incubated for 4 min at 37 °C. The absorbance was read at 593 nm using a spectrophotometer (Accuris MR9600-E, SmartReader, 230V) relative to water as blank. Trolox (250 uM) was used as an internal control. The results were expressed as µmol ferrous sulfate equivalents (FSE) per gram of samples using a linear standard curve generated with different concentrations (50–750 mM) of $FeSO_4.7H_2O$. Measurements were conducted in triplicate.

2.3.4.3 Total Soluble Protein

Frozen samples (5 g) were pulverized for 1 min using a metal roller (Waring Laboratory, Torrington, CT, USA) containing 45 ml of freshly prepared phosphate buffer saline of pH 7.4. The homogenate was stirred for 30 min and then centrifuged at 15000 rpm for 15 minutes. The supernatant was then collected into a new tube and quantified using Lowry's method, according

to (Shen et al., 2013). After 60 minutes, the absorbance of the samples was read at 700 nm with a spectrophotometer (BioTek Instruments, Winooski, VT, USA) against a BSA standard curve. The soluble protein content of samples was expressed as milligrams of Bovine Serum Albumin (BSAE) per gram of fresh weight. Each sample was measured in triplicate.

2.3.4.4 Mineral Nutrient Analysis

Wild blueberry samples were dried, pulverized, and sent to the Plant and Soil Testing Laboratory, University of Maine, Orono, for mineral nutrient analysis. Briefly, cooled ashed samples were dissolved in 50 % HCl on a hotplate and brought to volume with Deionized water. Samples were analyzed by inductively coupled plasma optic emission spectrometer (ICP-OES; Thermo-Jarrell Ash model IRIS 1000 dual-view, Franklin, MA, USA) for N, Ca, K, Mg, Al, Bo, Cu, Fe, Zn, P, and Mn determination according to the methods previously described by Kaltra and Maynard (1991).

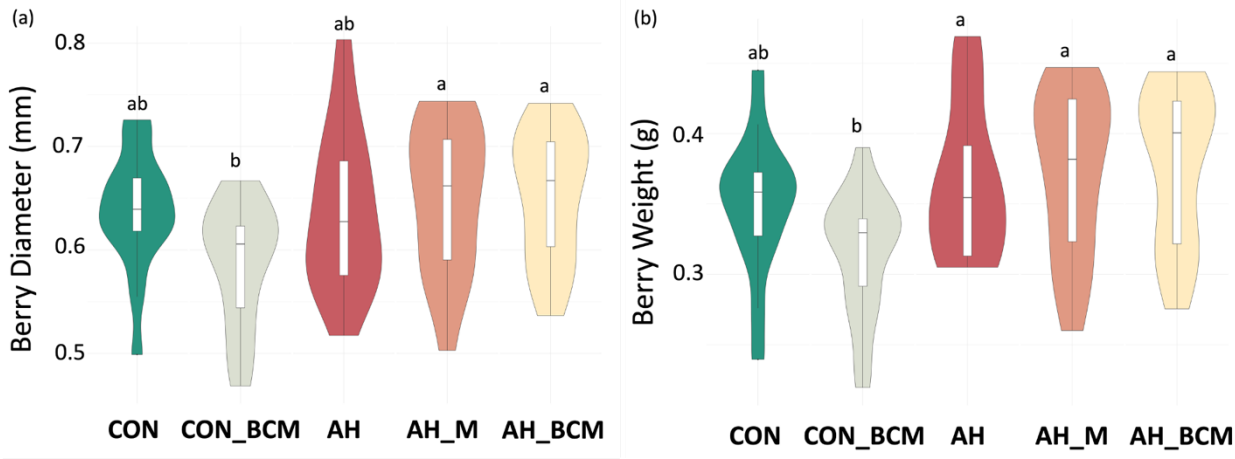
2.3.5 Statistical Analysis

The location and genotypes were treated as random factors while the treatments were fixed. The data collected were subjected to a two-way ANOVA after checking for normality by Shapiro-Wilk. Fisher's LSD mean separation test was conducted at $p < 0.05$. Pearson’s coefficient correlation analysis was conducted among berry quality traits (pH, TSS/TA (ripening index)), physical traits (size, weight, water content), metabolites (TPC, TAC, TSP, and organic acids), and antioxidant activities (FRAP).

2.4 Results

2.4.1 Berry Physical Properties

The berry diameter and weight showed no significant differences between control plots (CON) and ambient plots with biochar-compost treatment (CON_BCM) (Figure 2.1a & 2.1b). No significant ($p > 0.05$) difference was found in berry size and weight among all warming plots, including warming plots with no amendment (AH), warming with mulching (AH_M), and warming with biochar-compost mix (AH_BCM) treatments (Figure 2.1a & 2.1b). The berry diameter of wild blueberries under AH_M and AH_BCM was significantly higher ($p < 0.05$) than that of CON_BCM (Figure 2a). the berry weight of wild blueberries under AH, AH_M, and AH_BCM was significantly higher ($p < 0.05$) than for CON_BCM (Figure 2.1b) . The water content of berries under AH_M and AH_BCM was significantly higher ($p < 0.05$) in than for CON_BCM, (Figure 2.1c).



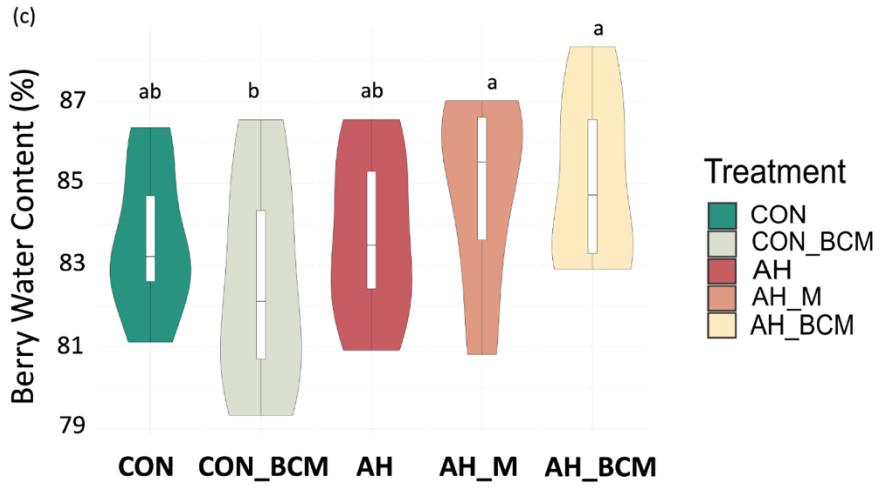


Figure 2.1: (a) Berry diameter (mm), (b) Berry weight (g), and (c) Berry water content (%) as wild blueberry physical properties under climate warming plots with no amendment (AH), warming with mulching (AH_M), and warming with biochar-compost mix (AH_BCM) treatments compared to the control plot with no biochar-compost mix (CON) and control plot with biochar-compost mix (CON_BCM). The bar in the middle of the violin box denotes the average per 20 berries. Letters topping the box indicate Fisher's LSD post hoc test following a two-way analysis of variance. All significance levels were tested at an alpha level of 0.05.

2.4.2 Berry Physicochemical Properties

The pH of berries produced under CON, CON_BCM, and AH treatments did not differ significantly ($p > 0.05$) (Figure 2.2a). However, a significantly higher berry pH ($p < 0.05$) was observed for those under warming with amendments (AH_M and AH_BCM) compared to the CON (Figure 2.2a). Berry titratable acidity (TA) content of wild blueberries under all warming treatments with and without soil amendments (AH, AH_M, AH_BCM) were significantly lower ($p < 0.05$) than for control (CON) (Figure 2.2b).

The total soluble solids of berries in CON_BCM plots (12.5 ^{o}Brix) showed no significant difference compared to that of CON (12.3 ^{o}Brix) plots ($p > 0.05$; Figure 2.2c). All warming plots with and without soil amendments showed significantly lower total soluble solids compared to CON and CON_BCM plots. The total soluble solids of berries in AH_M and AH_BCM plots

were significantly lower ($p < 0.05$) compared to that of AH (Figure 2.2c). The TSS/TA (ripening index) in warming plots (AH and AH_BCM) were significantly ($p < 0.05$) higher than in the ambient controls (CON and CON_BCM) (Figure 2.2d).

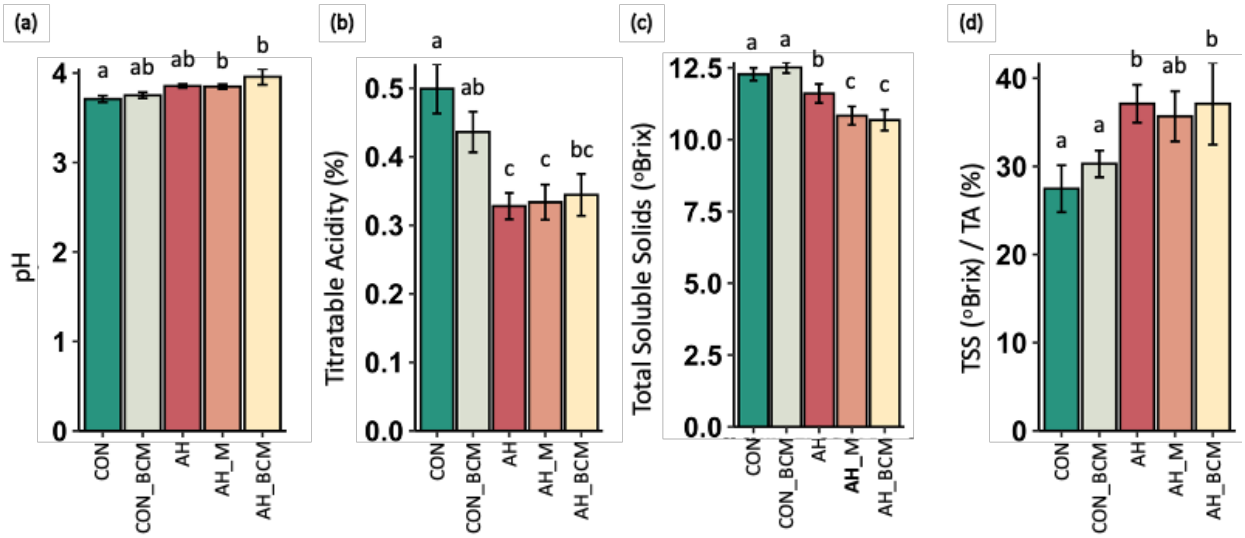


Figure 2.2: The pH (a), titratable acid (TA) (b), total soluble solids levels (TSS) (c), and the ripening index (the ratio of total soluble solids to titratable acidity) (d) in wild blueberry fruit under different warming conditions and mitigation treatments. CON: control without amendment treatment; CON_BCM: control with biochar-compost mix amendment treatment; AH: Active heating without amendment treatment; AH_M: Active heating with mulching amendment treatment; AH_BCM: Active heating with biochar-compost mix amendment treatment. Bars represent the means and standard errors of six replicated clones. Clones and locations were treated as random effects, while treatments as fixed effects. Bars showing the different letters significantly differ.

2.4.3 Organic Acids Composition And Concentration

Citric, quinic, gluconic, and shikimic acids were the primary organic acids in wild blueberries. Other organic acids, such as isocitric and malic acids, were below the detectable threshold and were dropped from further analysis. Citric, gluconic, and quinic acids were the most abundant organic acid in the wild blueberries (Figure 2.3). Citric acid was significantly lower ($p < 0.05$) in berries in AH compared to CON plots, while AH_M and AH_BCM and CON_BCM plots showed no significant differences (Figure 2.3a). Quinic acid concentration of berries under all warming treatments (AH, AH_M, and AH_BCM) were significantly lower ($p <$

0.05) compared to that of the CON and CON_BCM plots (Figure 2.3b). Gluconic and shikimic acids showed no statistical differences among treatments (Figure 2.3c & 2.3d). The total organic acid concentration of wild blueberries was significantly lowered ($p < 0.05$) in warming plots (AH, AH_M, and AH_BCM) compared to the CON and CON_BCM (Figure 2.3e).

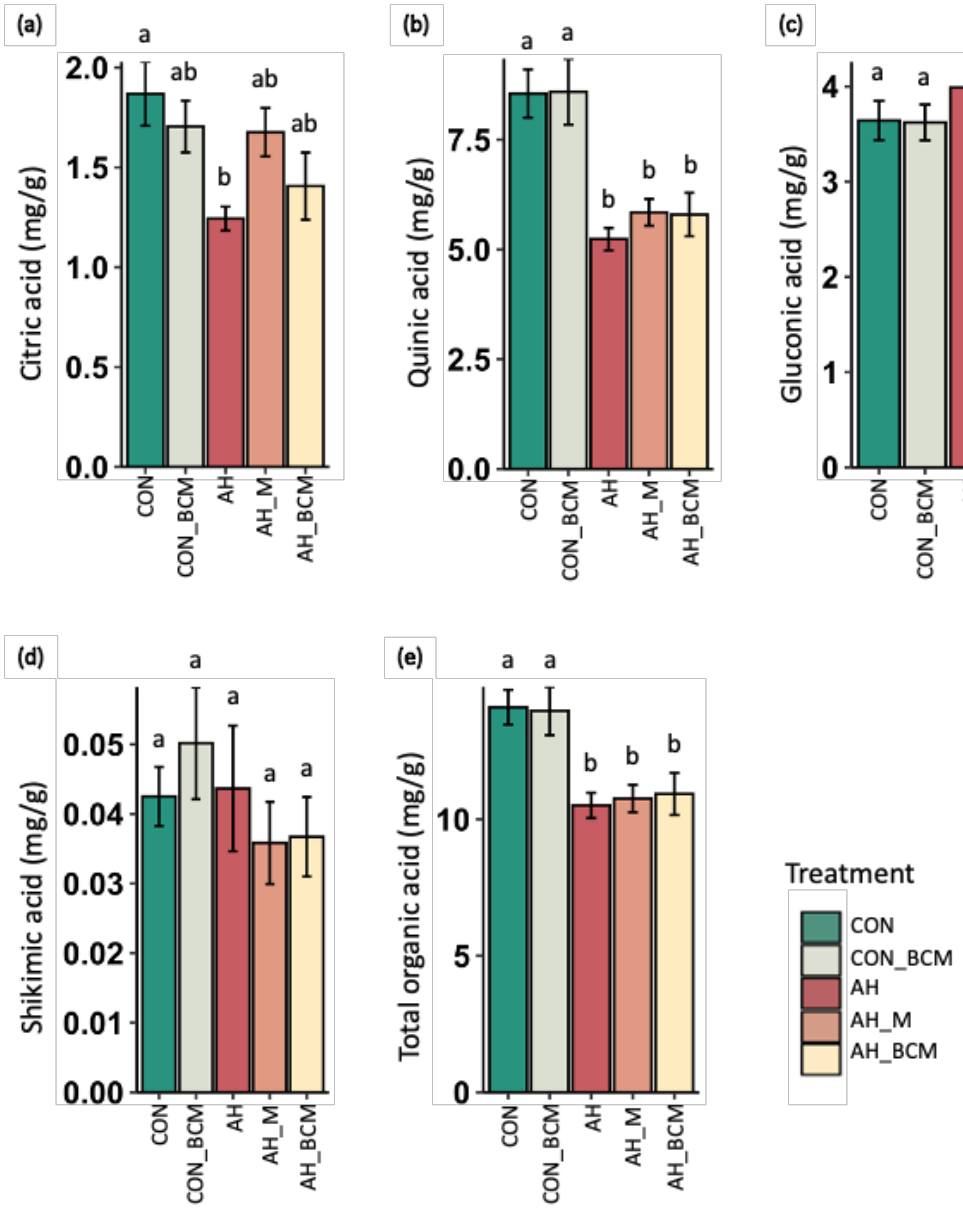


Figure 2.3: The citric (a), quinic (b), gluconic (c), shikimic (d), and total organic acids (e) in wild blueberry fruit under different warming conditions and mitigation treatments. CON: control without amendment treatment; CON_BCM: control with biochar-compost mix amendment treatment; AH: Active

heating without amendment treatment; AH_M: Active heating with mulching amendment treatment; AH_BCM: Active heating with biochar-compost mix amendment treatment. Bars represent the means and standard errors of six replicated clones. Clones and locations were treated as random effects, while treatments as fixed effects. Different letters are significant at an alpha level of ($p < 0.05$).

2.4.4 Soluble Sugars

The berry fructose concentration did not differ significantly in the AH plot compared to the CON plot (Figure 2.4a). However, the berry fructose concentration was significantly lower ($p < 0.05$) in the AH_M and AH_BCM plots compared to the AH plot. Berry glucose concentration in AH_BCM plots was significantly lower ($p < 0.05$) than those in AH_M plots (Figure 2.4b). However, berry glucose concentration was not different among the CON, CON_BCM, AH, and AH_BCM plots (Figure 2.4b). The total soluble sugar concentration showed no significant ($p > 0.05$) difference among CON, CON_BCM, AH, and AH_BCM plots (Figure 2.4c). However, berry total soluble sugar concentration in AH_BCM plots was significantly lower ($p < 0.05$) than those in AH_M plots (Figure 2.4c).

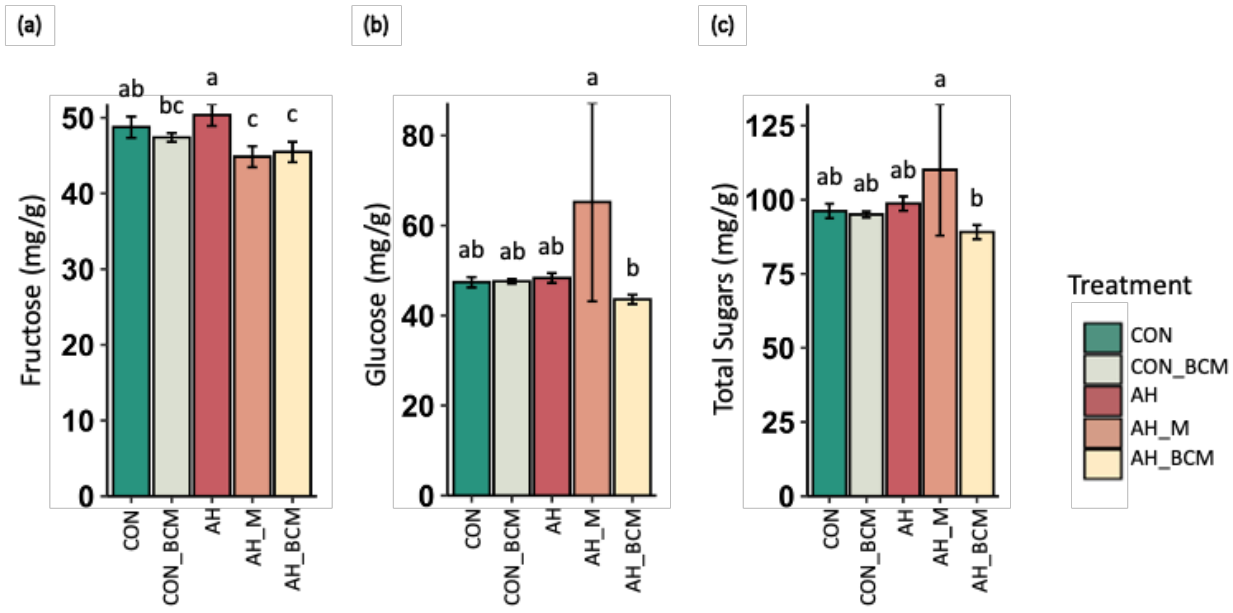
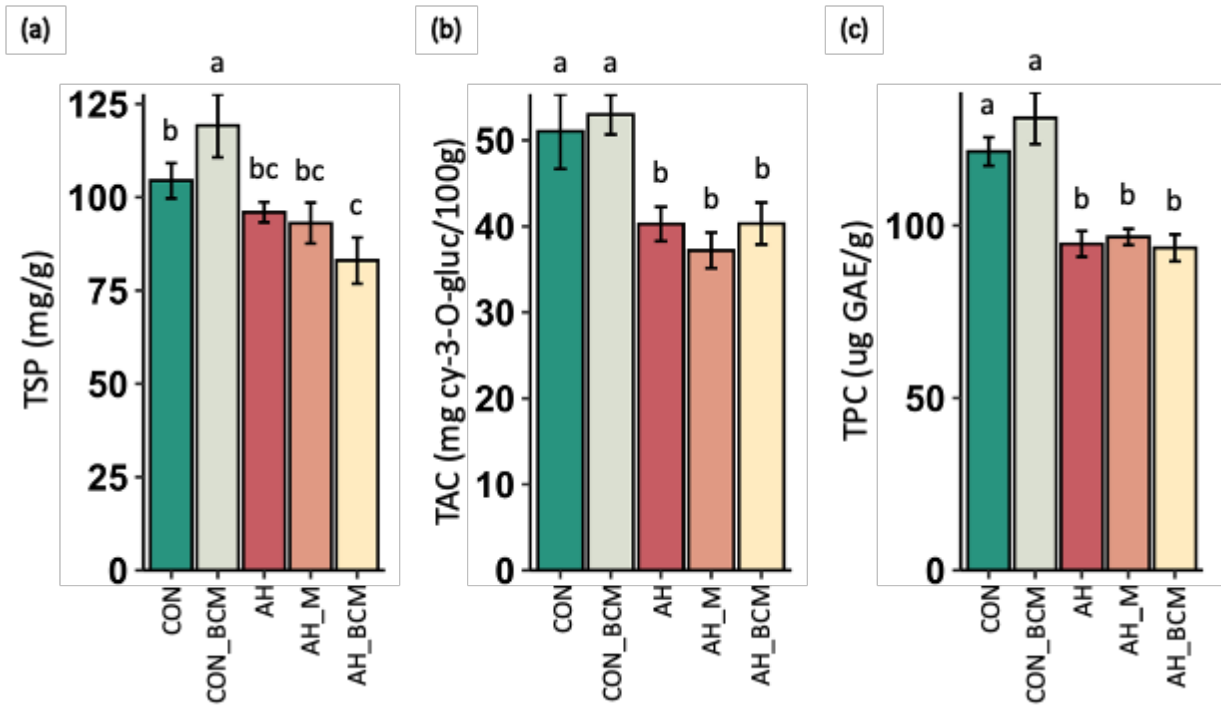


Figure 2.4: The fructose (a), glucose (b), and total soluble sugars (c) in wild blueberry fruit under different warming conditions and mitigation treatments. CON: control without amendment treatment; CON_BCM: control with biochar-compost mix amendment treatment; AH: Active heating without amendment treatment; AH_M: Active heating with mulching amendment treatment; AH_BCM: Active

heating with biochar-compost mix amendment treatment. Bars represent the means and standard errors of six replicated clones. Clones and locations were treated as random effects, while treatments as fixed effects. Different letters are significant at an alpha level of ($p < 0.05$).

2.4.5 Secondary Metabolites And Antioxidant Properties

The total soluble protein (TSP) of wild blueberries was significantly higher ($p < 0.05$) in CON_BCM compared to CON (Figure 2.5a). TSP was significantly ($p < 0.05$) lower in AH, AH_M, and AH_BCM when compared to CON_BCM (Figure 2.5a). Moreover, TSP was significantly lowered in AH_BCM when compared to CON plots (Figure 2.5a). As regards to total anthocyanin (TAC), phenolic acid (TPC) and antioxidant activities (FRAP), no significant difference was found between CON and CON_BCM plots (Figure 2.5b, c & e). However, TAC, TPC, and FRAP were significantly ($p < 0.05$) lower in all warming-treated plots (AH, AH_M, and AH_BCM) compared to the CON and CON_BCM plots (Figure 2.5b, c & e). TAC/TPC ratios showed no significant differences among the treatments (Figure 2.5d).



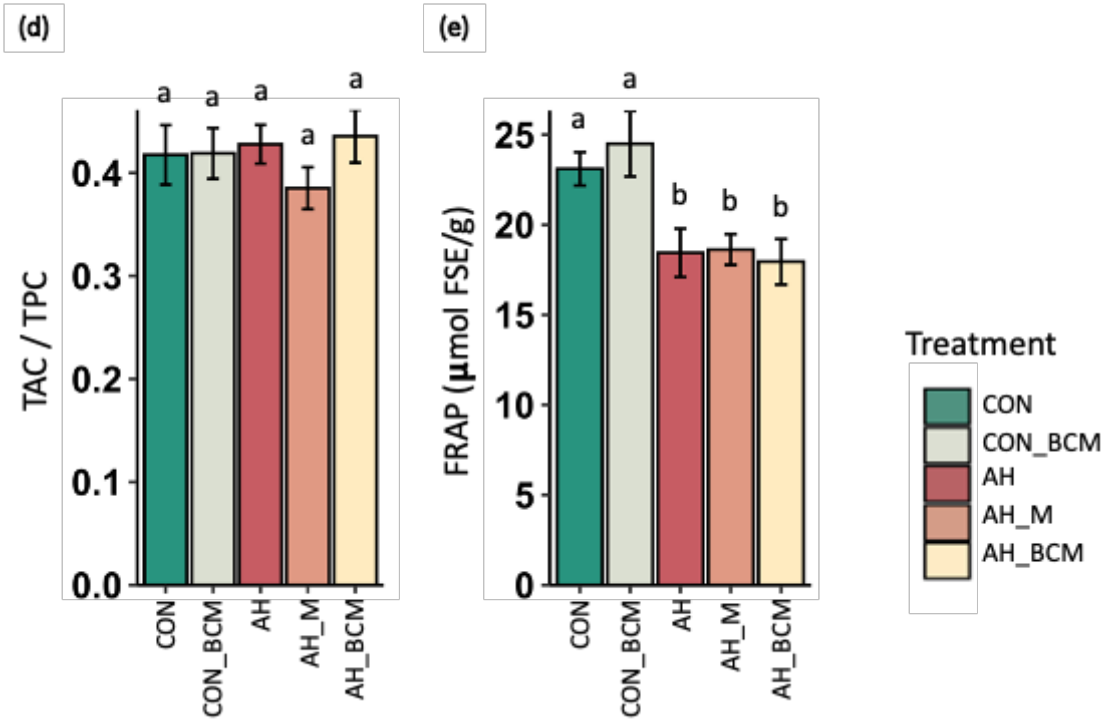
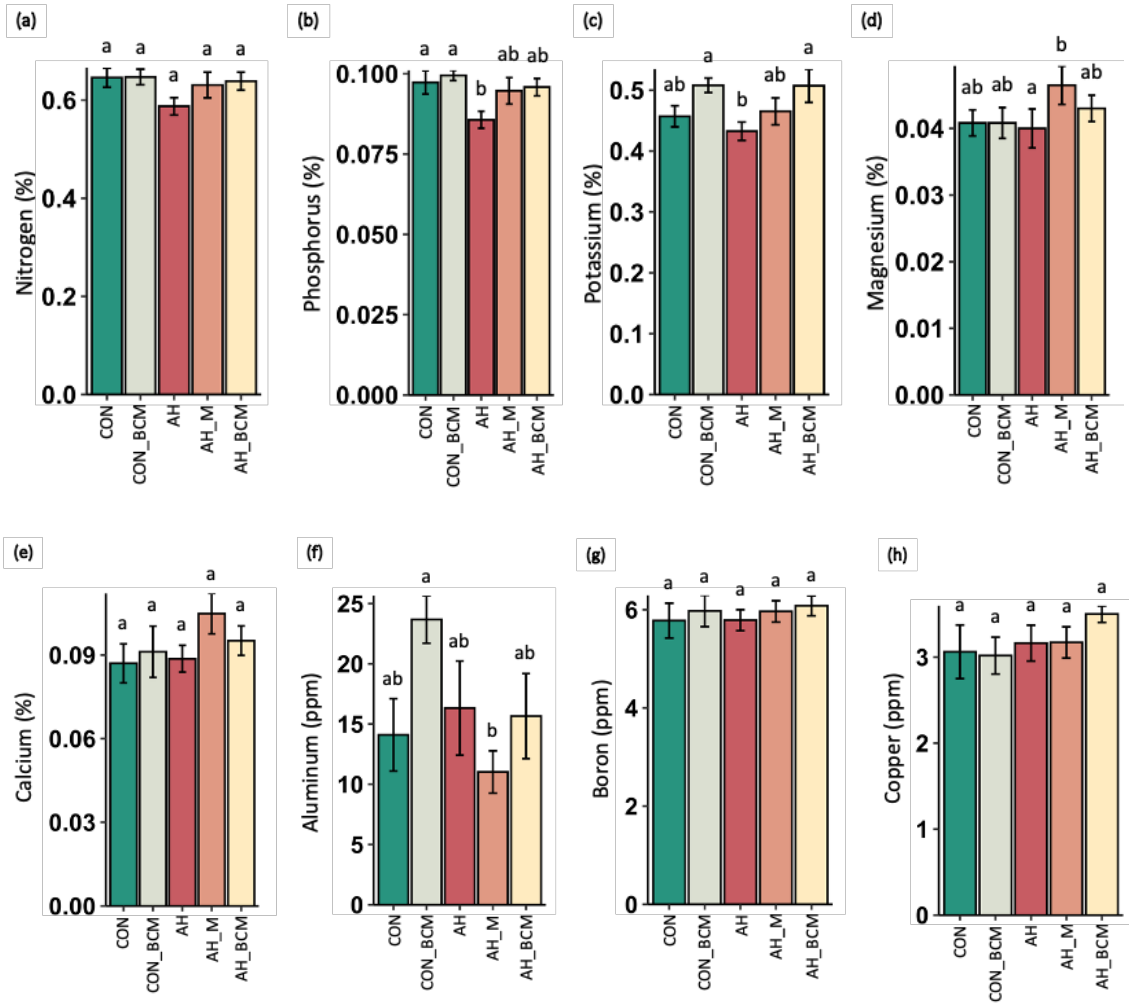


Figure 2.5: Total soluble protein (TSP) (a); total anthocyanin (b), total phenolic concentration (c), the ratio of total anthocyanin to phenolic concentration (TAC/TPC) (d), and ferric-reducing antioxidant power (FRAP) (e) from fresh-weight of wild blueberries under different warming conditions and mitigation treatments. CON: control without amendment treatment; CON_BCM: control with biochar-compost mix amendment treatment; AH: Active heating without amendment treatment; AH_M: Active heating with mulching amendment treatment; AH_BCM: Active heating with biochar-compost mix amendment treatment. Bars represent the means and standard errors of six replicated clones. Clones and locations were treated as random effects, while treatments as fixed effects. Different letters are significant at an alpha level of ($p < 0.05$).

2.4.6 Macro And Micro Minerals

Among treatments, no significant differences were observed in boron, copper, iron, manganese, and zinc concentration (Figure 2.6a, b,c,e, & f). The aluminum concentration was significantly ($p < 0.05$) higher in CON_BCM (23.5 ppm) than in AH_M (11 ppm) (Figure 2.6d). The berry calcium and nitrogen concentrations showed no statistical differences among treatments (Figure 2.6g & j). The lowest potassium concentration was found in AH plots and was significantly ($p < 0.05$) lower compared to that of AH_BCM plots (Figure 2.6h). The magnesium concentration was significantly ($p < 0.05$) higher in AH_M (0.045 %) than in AH plots (0.038

%) (Figure 2.6i). Berry phosphorus concentration was significantly higher ($p < 0.05$) in CON and CON_BCM plots than AH plots.



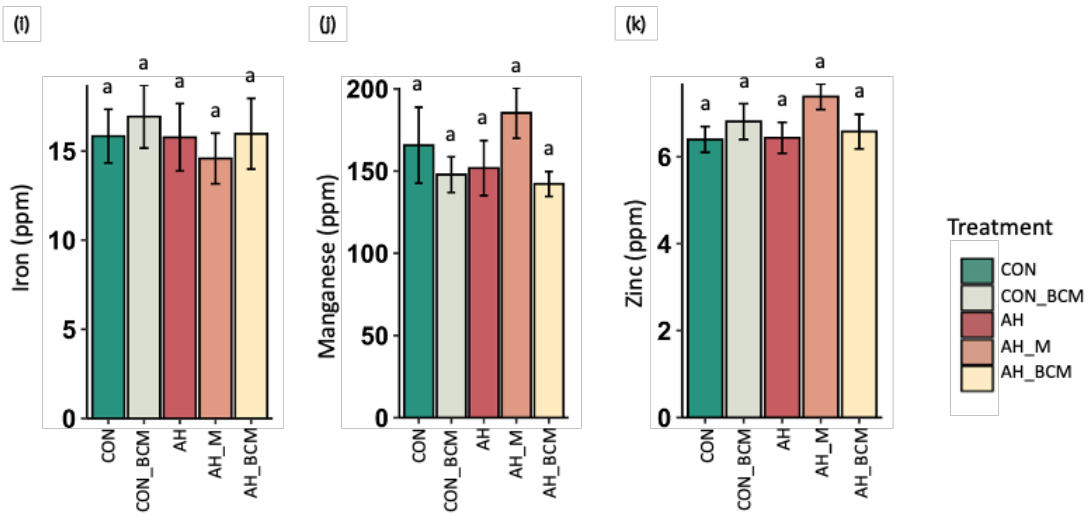


Figure 2.6: The average concentration of essential microminerals and macrominerals: iron (a), manganese (b), zinc (c), aluminum (d), boron (e), copper (f), calcium (g), potassium (h), magnesium (i), Nitrogen (j) and phosphorus (k) in mature wild blueberries under different warming conditions and mitigation treatments. CON: control without amendment treatment; CON_BCM: control with biochar-compost mix amendment treatment; AH: Active heating without amendment treatment; AH_M: Active heating with mulching amendment treatment; AH_BCM: Active heating with biochar-compost mix amendment treatment. Bars represent the means and standard errors of six replicated clones. Clones and locations were treated as random effects, while treatments as fixed effects. Different letters are significant at an alpha level of ($p < 0.05$).

2.4.7 Correlation Between Wild Blueberry Quality Traits, Metabolites, And Antioxidant Activities

The berry pH was correlated positively with TSS/TA ratio, an index for berry maturity or ripeness (Figure 2.7). Conversely, pH and TSS/TA were negatively correlated ($p < 0.05$) with titratable acidity (TA). Berry size (diameter) and weight were positively correlated to berry water content while negatively correlated to total organic acids, total soluble protein, total sugars, TSS, total anthocyanin, and phenolic acids concentrations (Figure 2.7). TSS was positively associated with total sugars, total organic acids, and phenolic acids, but did not exhibit any significant correlation with total anthocyanin (Figure 2.7).

Gluconic acids (a derivative of glucose) did not show any significant correlation with total organic acids but interestingly correlated positively with glucose (Figure 2.7). Soluble

protein (complex organic compounds made up of amino acids) was positively associated with shikimic, phenolic acids and antioxidant activities (FRAP). Interestingly, antioxidant activities (FRAP) exhibit a strong positive correlation with phenolic acids and a weak positive correlation with anthocyanins (Figure 2.7).

To gain additional insight into relationships of the quality traits, physical characteristics, and metabolites, a hierarchical clustering (HC) was conducted. These parameters were grouped into five clusters (C1-C5) (Figure 2.7). These five groups include the fruit maturity or ripeness parameters (cluster 1), berry physical traits (cluster 2), anthocyanins (cluster 3), total soluble solids and sugar-related compounds (cluster 4), and phenolic acids, soluble proteins, and organic compounds (cluster 5).

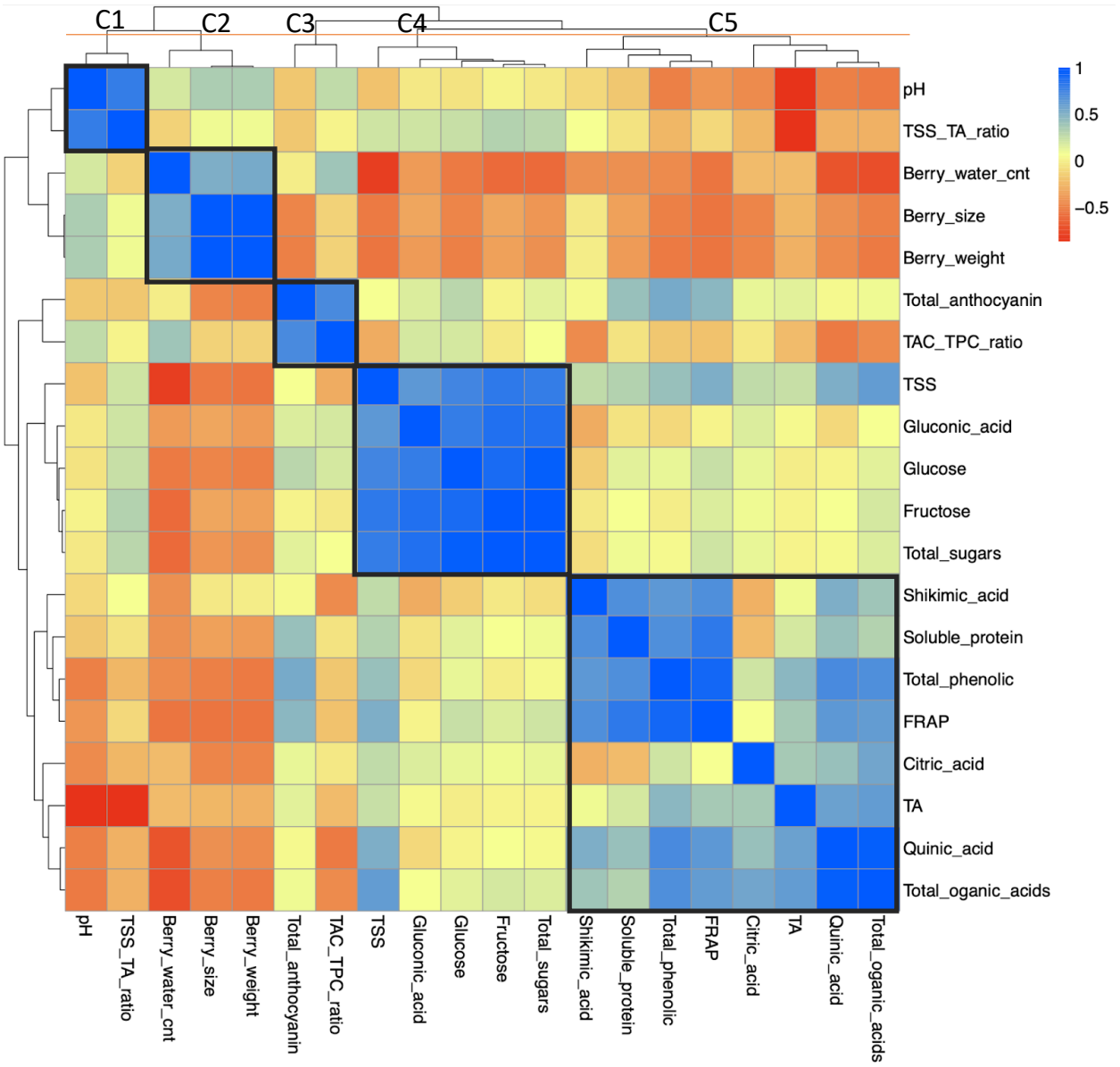


Figure 2.7: Correlation analysis of nutritional quality traits, metabolites, and antioxidant activities of wild blueberry. The light-yellow boxes represent trait with no correlations. Traits displaying cyan to blue colors indicate positive correlations, while yellow to red indicate negative correlations. Berry pH and ripeness index (TSS/TA ratio) grouped in the cluster 1 (C1); berry water content, size and weight grouped together in cluster 2 (C2); anthocyanins and TAC/TPC grouped in cluster 3 (C3); total soluble solids, fructose, glucose and sugar-related compounds grouped together in cluster 4 (C4); and phenolic acids, antioxidant activities (FRAP), soluble proteins, titratable acidity, and total organic acids, citric acids and quinic acids grouped together in cluster 5 (C5).

2.5 Discussion

Our study confirms the negative effects of climate warming on the nutritional quality of wild blueberries, which have high health benefits. Although biochar or mulching increased berry potassium and magnesium concentrations, our hypothesis that mulching and biochar-compost mix amendments in wild blueberry soils will reduce the negative effect of warming on fruit quality was not supported. The major organic acids and soluble sugars found in this study agree with previous research. We found that the most abundant organic acids in wild blueberries are citric, quinic, gluconic, and shikimic, while malic and isocitric acids were below detectable levels, which agrees with another study on wild blueberries (Kalt and McDonald 1996). We detected fructose and glucose, but not sucrose, in wild blueberries using HPLC, which fully agrees with a previous study (Kalt and McDonald 1996).

Negative effects of warming on the nutritional quality of wild blueberries

This current study showed a consistent reduction in total soluble protein and soluble solids of wild blueberries under climate warming, which is similar to what was found in our previous study (Alaba et al. unpublished). Specifically, total soluble protein in wild blueberries was reduced under elevated temperatures (passive warming and active warming) compared to ambient temperatures. This agrees with previous studies that found lowered soluble protein concentration in rice and wheat under warming (Chang-lian et al. 2005; Zhao et al. 2007). This effect of climate change may be due to the sensitivity of proteins, including enzymes, to heat stress and elevated temperatures (Ebi and Loladze 2019). Moreover, CO_2 enrichment can also cause declined wheat protein concentrations (DaMatta et al. 2010; Taub et al. 2008), which will exacerbate the negative effect of warming. Decreased total soluble solids under elevated temperatures in wild blueberries are also consistent with other previous studies (Hopkirk et al.

1989; Juszczak et al. 2010; MacKenzie et al. 2011; Menzel 2022; Alaba et al. unpublished). Increasing growth stages and faster fruit maturation under elevated temperatures may be accompanied by decreasing total soluble solids (Lopresti et al. 2014; Menzel 2022). In contrast, TSS in apples increased under elevated temperatures, although not statistically significant (Sugiura et al. 2013). In this study, fructose concentration did not respond significantly to treatments under warming plots with no amendment (AH) compared to ambient control (CON). This contradicts the result observed in our previous experiment (Alaba et al. unpublished), where fructose concentration was reduced.

In this study, phosphorus mineral concentrations, which aid metabolic mechanisms such as energy transfer, were significantly reduced under elevated temperatures compared to ambient conditions. Under elevated temperatures, phosphorus mineral concentrations are predicted to decrease due to a significant correlation between total soluble solids and phosphorus mineral concentrations Guedes et al. (2013). A few studies have reported the negative impact of high temperatures on macrominerals and microminerals, which may be through water stress induced in crops (Soares et al. 2019; Giulia et al. 2020). Another study indicated the positive effect of direct sunlight exposure on increasing the macro minerals such as potassium, magnesium, and calcium requirement in avocados (Woolf et al. 1999).

Elevated temperatures also reduced the total anthocyanin and phenolic acids concentrations and, consequently, the antioxidant activity in wild blueberries compared to that under ambient temperatures. Biochemical and transcriptomic analysis revealed that many genes in the flavonoid biosynthesis pathways were down-regulated under elevated temperatures (Lecourieux et al. 2017), reducing anthocyanin and flavonoid contents in major fruit crops such as grapes, red-fleshed kiwifruit, and strawberries (Barnuud et al. 2014; Gouot et al. 2019; Man et

al. 2015; Matsushita et al. 2016). However, in our previous study, total anthocyanin, phenolic acids, and flavonoids in wild blueberries did not change under elevated temperatures compared to ambient conditions (Alaba et al., unpublished). The differences in treatment response of wild blueberry secondary metabolites from the two experiments may be due to seasonal differences. In strawberries grown under elevated temperatures showed higher anthocyanin, flavonoid, and antioxidant properties (Wang and Zheng 2001). The effect of elevated temperatures on secondary metabolites in fruits may be genotype and plant dependent.

Limited impact of soil amendments on berry nutritional quality

Overall, two years of mulching and biochar-compost mix treatment could not mitigate the negative effect of warming on the nutritional quality of wild blueberries. Biochar-compost mix, and mulching tended to mitigate the effect of warming on phosphorous, potassium, and magnesium. This finding agrees with a previous study that found higher K, P, and Mg in kiwi fruits with biochar treatment in soil (Sorrenti 2015), as biochar tends to release these macro minerals on the soil surface. Our results showed that soil amendments like mulch and biochar-compost did not affect citric, quinic, and total organic acid, which contradicts findings from previous studies that suggested that biochar may aid the storage of organic acids in berries (Sani et al. 2020; Wang et al. 2014). Biochar-compost mix and mulching also failed to exert any significant effects on the fructose content of berries in this study. Like our findings, wood-based biochar did not significantly influence the total soluble sugars in grape and tomato fruits, respectively (Schmidt et al. 2014; Petrucceli et al. 2015).

Under ambient conditions, the biochar compost treatment (CON_BCM) significantly increased the total soluble protein in wild blueberries compared to CON, suggesting the positive effects of the biochar-compost mix. However, the amount of biochar-compost mix applied in the

warming plots may not be sufficient to mitigate the negative effect of elevated temperatures on soluble protein. Biochar-compost mix, and mulching did not mitigate the detrimental impacts of high temperatures on secondary metabolites, including anthocyanins, indicating that the future climate might significantly reduce the health-promoting benefit of wild blueberries (Debnath-Canning et al. 2020). Furthermore, biochar-compost mix, and mulching showed no significant effect on the concentration of microminerals, indicating that these requirements were met even under elevated temperatures.

Biochar or mulching did not significantly mitigate the effect of warming on TA and TSS that influence the sweet taste of wild blueberries. Contrary to our findings, biochar amendment positively influenced the TA and TSS of tomatoes grown under field conditions (Caliman et al. 2010). Furthermore, our result showed that the biochar-compost mix maintained the TSS/TA ratio of wild blueberries under warming, suggesting that the biochar-compost mix may retain the ripeness and flavor qualities of wild blueberries under elevated warming or stressful conditions. Specifically, the TSS/TA in tomatoes grown under drought conditions was maintained by adding biochar (Akhtar et al. 2014). The TSS/TA ratio was used as a ripening index, maturity, or flavor for blackberries, blueberries, and many other fruit crops (Milošević et al. 2012; Mengist et al. 2020).

Berry size and pH in relation to berry nutritional quality

We found a positive relationship between berry pH and TSS/TA (maturity and ripeness index), which suggests that berry pH may associate with berry ripeness and maturity. Our result agrees with a previous study on highbush blueberries (*Vaccinium corymbosum*), suggesting that ripeness could be a pH-dependent process (Mengist et al. 2020). Ripe desert melon (*Cucumis*

melo L.) has been reported to show recessiveness to the genetic trait of sourness, or acidity (PH genes), thereby making the fruit taste sweet (Cohen et al. 2014).

We found negative relationships between berry size (diameter or weight) and total anthocyanin or phenolic acid concentrations, suggesting that smaller-sized berries have more anthocyanin and phenolic acids contributing to the antioxidant properties than larger-sized berries. Previous studies indicated that berry skin stores secondary metabolites (Ma et al. 2021). Thus, smaller berries could show higher concentrations of secondary metabolites because of their higher surface area to volume ratio. This result also showed that berries with bigger sizes tend to have lower total soluble solids as there appear to be negatively correlated. The effect of BCM and mulching amendment on the wild blueberry fields under warming did not significantly increase the average berry size. Similar to our finding, there was no difference in the weight and size of tomatoes grown under the biochar soil amendments (Petruccelli et al. 2015). Berry size and weight correlated positively with berry water content. Higher water content status has been linked to larger berry size and expansion in grapes (Fouquet et al. 2008). On the other hand, the increased berry size could be a water conservation strategy the crop adopts to withstand the warming stress. In a previous study, elevated temperatures enhanced the wild blueberry sizes and weight (Chen et al. 2022), which could be attributed to the rate of water movement or evapotranspiration in the wild blueberry crop.

2.6 Conclusion

To conclude, this study reinforced the detrimental impact of elevated temperatures on the nutritional quality attributes of wild blueberries and found that soil amendments such as biochar-compost mix and mulching, at least the rate we tested, could not mitigate the impact of warming on the nutritional qualities of wild blueberries, except for potassium and magnesium

concentrations. It is possible that the negative impact of warming on the nutritional quality is due to the temperature effect on metabolism and could not be mitigated by enhancing soil water and nutrient supply. Therefore, whether increasing the application rate of biochar-compost mix and mulching or other agricultural approaches that can mitigate the expected negative effect of warming on crop nutritional qualities need further testing. Physiological and biochemical mechanisms need to be better understood to better explain the warming impact on berry nutrition. Possible techniques for enhancing berry nutritional quality need to be tested to maintain the high nutritional quality of wild blueberries in a warmer climate.

CHAPTER 3

WARMING TREATMENTS SHIFT THE DIVERSITY AND COMPOSITION OF BACTERIA IN WILD BLUEBERRY SOILS IN THE EARLY GROWING SEASON

3.1 Abstract

Soil bacterial communities are an essential biological indicator of soil health and crop performance, but how they will respond to climate change is not well understood. In Maine, wild blueberry (*Vaccinium angustifolium*) farms are experiencing unprecedented temperature changes, which could aggravate microbial responses and may harm the crop. To understand how bacterial communities respond to warming during the growing season, we used passive and active open-top chambers to simulate climate warming scenarios in wild blueberry fields. Warming treatments elevated atmospheric temperatures by 1.2 and 3.3 ^{o}C (passive and active warming), but not soil temperatures. However, soils in the active warming treatment showed significantly lower water content than the ambient. Overall, soil bacteria diversity and richness (June, July, and August data combined) under the warming (passive and active) treatments and ambient controls did not show significant differences after experimental warming for two years. However, significantly higher bacterial evenness and diversity under warming treatments were found in the early growing season (June). Our study also shows strong seasonal shifts in the evenness and diversity of bacteria in the wild blueberry soil, suggesting that the variation in bacterial community structure may be more influenced by seasonal changes in temperature and plant activity during the growing season than by warming treatments. The increased bacteria evenness and diversity under warming treatments in June could be related to advanced plant phenology, suggesting a future shift of seasonal dynamics in bacterial activity under global warming.

3.2 Introduction

The immense benefits of soil microbes in agriculture make them an essential biological indicator of soil health and crop performance. Soil microorganisms coexist and interact closely with the roots of crops to obtain sugars and organic acids from root exudates (Clark, 1949; Kumar et al., 2007). Additionally, soil microorganisms play a significant role in ecosystem processes through their symbiotic interactions, including depolymerizing organic compounds, recycling nutrients into forms that plants can easily absorb, and enhancing plant tolerance to environmental stresses (Burges, 1967; Yuan et al., 2018; Khan et al., 2019; Shah et al., 2021; Prasad et al., 2021). These fundamental roles highlight the importance of soil microbes in promoting plant health and performance. Farming management practices such as fertilization, mulching, tillage, and cropping systems have been observed to alter soil microbial diversity and composition of forests and farms (Ishaq, 2017; Madegwa & Uchida, 2021; Ishaq et al., 2020; Yeboah et al., 2016). Other factors impacting the diversity and composition of soil microbial communities in any ecosystem include soil pH (Zhalnina et al., 2015), vegetation (Ravit et al., 2006; Hui et al., 2017), heavy metals (Kandeler et al., 1996), land use (Kuramae et al. 2012), and soil water content (Brockett et al., 2012; Schimel, 2018). Recently, global attention has been drawn to the negative impact of climate change on soil microbe communities and possible mitigation strategies that could improve soil health (Zogg et al. 1997; Mandal and Neenu 2012; Ishaq et al. 2020).

The annual mean surface atmospheric temperatures have increased by nearly 1.1°C and are projected to increase by 5.4°C by the end of this century if greenhouse gas emissions stay at the current frequency (IPCC, 2014). These accelerated temperature increases are critical factors that can impact soil microbial communities but have not been well-investigated to reveal a

general pattern. Elevated atmospheric and soil temperatures can also decrease soil moisture content due to enhanced evapotranspiration, negatively affecting critical plant-soil microbial interactions (Rasmussen et al., 2020). In temperate regions, two decades of continuous experimental warming have been reported to significantly change the bacteria community composition (DeAngelis et al., 2015). In some cases, seasonal changes, rather than warming, caused a substantial alteration in soil microbial community composition (Madegwa & Uchida, 2021; Pold et al., 2021). Climate warming expanded the differences in initial soil properties of natural forests and plantations, resulting in different responses in microbial communities in a short-term experiment (Zhao et al. 2022). Meanwhile, certain bacteria genera, such as *Pseudomonas* and *Bacillus,* can promote plant yield and quality under water stress (Nordstedt & Jones, 2020; Paliwoda et al., 2022), attributing to the diverse functions of microorganisms inhabiting the soil. *Acidothermus*, a heat loving *Actinobacterium*, were also observed to recycle plant biomass faster in warming environments (Viitamäki et al. 2022). The incidence of soil-borne pathogens was noted to increase under elevated temperatures, threatening plant health and production (Delgado-Baquerizo et al. 2020). As the surface temperature increases, the roles of soil microbes and their interactions with perennial crops in ensuring food security and human nutrition remain exciting but unanswered research questions (Leisner, 2020).

Wild blueberry (mainly *Vaccinium angustifolium*) is a perennial fruit crop native to North America. This economically important crop has a 2-year cropping cycle, with harvested berries occurring every other year. Its rhizomes form natural carpets that host diverse microorganisms within a thin layer above the sandy soil (organic pad) with a pH of 4 (Yarborough, 1996). These plant-microbe interactions in the wild blueberry ecosystem foster crop growth, yield, and fruit nutritional quality (Ramasamy et al., 2011; Li et al., 2020). The wild blueberry provides an ideal

system to study the effect of warming on the bacteria community because wild blueberries lack long root hairs, and their growing fields are experiencing abnormal climate warming and water stress (Barai et al., 2021; Tasnim et al., 2021; Chen et al., 2022). Unprecedented environmental warming and erratic seasonal patterns associated with climate change have been observed on wild blueberry fields (Tasnim et al., 2021). Climate warming alters the phenology, morphology, and physiology of wild blueberries (Chen et al., 2022), while the economic revenue of Maine and Canada, the primary producers of wild blueberries globally, is expected to be challenged by the detrimental effects of climate warming (Yarborough, 1997; Drummond et al., 2009). Previous studies have focused on the above-ground response of wild blueberries to climate warming (Tasnim et al., 2021; Chen et al., 2022; Alaba et al., unpublished). Many studies revealed that warming shifted soil microbial diversity and activity, suggesting that it could play roles in above-ground plant growth and yield (Zhang et al., 2005; Luo & Weng, 2011; Lladó et al., 2017; Chen et al., 2020). Therefore, the response of belowground microbial communities to the current unprecedented climate warming on the wild blueberry terrestrial ecosystem needs to be investigated.

This study aimed to profile the bacterial community composition and diversity of wild blueberry fields under climate warming scenarios and ambient conditions *in situ*, thereby providing the basis for potential soil management strategies that can sustainably lessen the impact of climate warming in the future. We used Illumina amplicon 16S rRNA gene sequencing to reveal the differences in the wild blueberry soil bacterial composition and abundance under the open-top chamber warming treatments over two years (2019 and 2020) of growing seasons (Sadras et al., 2012). We hypothesized that elevated atmospheric temperatures of 1.2 ^{o}C and 3.3 ^{o}C for two years would alter the soil temperatures, reduce the soil water content and shift soil

microbial community composition in warming chambers compared to ambient control plots. Also, since the summer is warmer than the late spring of the growing season, we predicted that microbial community composition at the beginning of the growing season (June) would differ from the end of the growing season (July and August) whether or not under warming scenarios. Our results provide insights into the response of the bacterial community to climate change, as well as the interactions between plant phenology and the activity of microbial communities.

3.3 Materials And Methods

3.3.1 Study Site And Experimental Design

This experiment was set up at the Blueberry Hill Research Farm in Jonesboro (44.644 N, 67.646 W) in 2019 and 2020. The wild blueberry experimental site comprises gravelly sandy loam soils of 4.7 pH and a humid continental temperate climate (Smagula and Hepler 1978; García-Gaines and Frankenstein 2015). The mean annual temperature of the study site is 6.9 ºC, and the mean annual precipitation is 1298 mm (NOAA, 2023). The study was set up in April 2019 before the re-sprouting (prune year) of wild blueberries from belowground biomass, while sampling was done in the growing season of the following crop year, 2020.

Open-top chambers were designed since April 2019 to simulate realistic warming scenarios (Tasnim et al. 2020). Both active and passive heating open-top chambers were designed with 55 cm high x 100 cm width dimensions slanted at 60 degrees on six sides to enclose wild blueberry plants using translucent polycarbonate sheets. The active heating (AH) chambers used heating tapes (Briskheat, Columbus, OH, USA) inside the chambers to continously raised the mean air temperature by 3.3 ºC for two years (2019 and 2020), while the passive (PH) chamber had no heating tapes and raised the mean air temperature by 1.2 ºC. The ambient condition (CON) has no open-top chamber. A randomized block design previously

described by Chen et al., (2022) with six genotypes was used. Briefly, six morphologically diverse wild blueberry (*V. angustifolium*) genotypes were randomly selected and used as blocks containing the three different temperature treatments: AH, PH, and CON. Weather stations raised to a height of approximately 10 cm above the soil, were installed in the middle of ambient plots and both active and passive open-top chambers. Detailed dynamics in mean atmospheric temperature and relative humidity during the spring and summer seasons of soil sampling are reported by Chen et al. (2022). Soil temperatures and soil volumetric water contents were determined using a TDR 150 Soil Moisture Meter (Spectrum Technologies Inc., Aurora, IL, USA) installed in the middle of the plots, and at a depth of 5 cm for this study. No fertilizer or irrigation were applied during the two years of study.

3.3.2 Soil Sampling And Processing

Soil sampling was carried out in the crop year 2020. The soil samples were collected on following dates in 2020 to account for seasonal differences: June 09 (late spring), July 19th (early summer), and August 18th (late summer). A soil probe of 3 cm diameter was used to collect three samples from the top 10 cm of soil along an imaginary triangular line at each treatment plot. The top of the organic matter was removed. The fresh core samples were homogenized thoroughly in the collection bags to make composite soil and then transported to the laboratory at the University of Maine. The composite soil samples were sieved using a 2-mm sieve to ensure roots and stones were removed before storing aliquots at -20°C for DNA extraction.

3.3.3 Soil DNA Isolation And Real-Time Quantitative PCR

DNA was extracted from 52 soil and control samples collected from the three experimental treatments of AOTC, POTC, and ambient (control) groups using previously curated

protocols. Soil samples diluted in sterile phosphate-buffered solution were homogenized and treated with propidium monoazide (PMA; BioTium) at a final concentration of 25 μM following the manufacturer's protocols. PMA covalently binds to DNA inside dead cell membranes or free DNA to prevents their DNA amplification in downstream analysis (Nocker et al. 2007).

Bulk DNA was isolated from the PMA-treated soil (n = 48 samples), or no-template (water) control samples (n = 4, one for each extraction batch) using Quick-DNA Fecal/Soil Kit optimized for soil-based microbial communities (Zymo Research, Freiburg, Germany). The quantity and purity of extracted DNA was determined using a Thermo Scientific™ NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, U.S.). The 16S rRNA gene V3-V4 region of the samples were amplified using 515F / 926R primers according to the protocols from The Earth Microbiome Project (Walters et al. 2016), and sequenced on an Illumina MiSeq platform using the 2 x 300-nt V3 kit (Molecular Research Labs, Clearwater, TX, U.S.).

The total copies of bacteria 16S rRNA in wild blueberry soil microbial communities were assessed using qPCR (Applied BiosystemsTM, Waltham, MA, USA). Following the manufacturer's protocol, the products were detected by fluorescence using a Luna Universal qPCR Master Mix Kit. Gene block standards purchased from Integrated DNA Technologies (IDT) were serially diluted from 10^6 to 10^1 copies. Amplification of template DNA from both samples and DNA from diluted standards was performed using the bacteria-specific primers F (1048F Bac) (Horve et al. 2020) and R (1194R Bac) (Horve et al. 2020). The reaction volume was 20 uL because 2 uL of genomic DNA, 0.5 uL of each primer, 10 uL of SYBR Green qPCR master mix (Ref Biolabs Inc.), and 7 uL of nucleic-free water were used. The bacterial 16S ribosomal RNA genic region, V3-V4, was amplified by using Applied Biosystems StepOne

(Applied Biosystems, Foster City, USA) with the following thermal cycling conditions: 95 °C for 2 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and 72 °C for 5 min.

The samples and each dilution in a standard curve were performed in triplicate. Based on the linear relationship between the mean cycle threshold (Ct) values and the logarithm gene copy number of standards, the number of gene copies in wild blueberry soil bacteria per uL of DNA was calculated.

3.3.4 Amplicon Sequencing Analysis

Raw Illumina sequencing datasets were processed using the DADA2 (version 1.16) pipeline, as Callahan et al. (2016) described in the R platform (version 4.1.3). The standard sets of parameters in DADA2 were followed to merge raw sequence pair-end reads, trim for quality assurance, denoise, dereplicate, and filter for chimeric sequences. The SILVA reference database (v138) was used to identify taxa for the representative amplicon sequence variants (ASVs) produced. ASVs obtained from the negative controls that were DNA extracted and sequenced were used to decontaminate the reads from soil samples. Unwanted sequence taxa classified as chloroplast and mitochondria were also cleaned out. The rarefaction curve was plotted and visualized in R. Samples were scaled with the lowest sequence reads of 13300 to even the depth, avoiding biases that could affect clustering analyses or diversity metrics. The ASV table and taxonomy file generated were used for microbial diversity and statistical analyses.

3.3.5 Microbial Diversity

Using the rarefied phyloseq object as described by McMurdie and Holmes (2013), we estimated Shannon indices (alpha diversity) from the number of observed bacterial ASVs (richness) and evenness. The normality in abundance and distribution of bacterial ASVs between

treatments were first assessed and tested using the Mann-Whitney-Wilcoxon non-parametric test ($p < 0.05$). Boxplot and correlation matrix were plotted to visualize the top 20 most abundant ASVs from the rarefied phyloseq object using the ggplot2 and corrplot packages in RStudio (Allaire, 2012). Beta diversity was studied based on Bray-Curtis's dissimilarity method. Constrained correspondence analysis (CCA) plots were performed for permutational multivariate variance analysis to compare the bacterial composition between warming treatment and seasonal change groups. A two-way ANOVA evaluated the significant effects ($p \leq 0.05$) of warming treatments and seasonal changes on soil bacterial composition and diversity, followed by a post-hoc analysis using Fisher's LSD mean separation ($P \leq 0.05$). All statistical analyses were performed in R using the RStudio 2022.07.1+554 platform (R Team; Allaire 2012). The raw sequence data were deposited in the NCBI SRA with Bioproject accession number: PRJNA925843.

3.4 Results

3.4.1 Environmental Conditions

Simulated experimental warming using the open-top chambers increased atmospheric temperature by 3.3 ^{o}C in the active warming chamber and 1.2 ^{o}C in the passive chamber than the ambient control plots during the wild blueberry growing seasons (Chen et al. 2022). However, warming treatments did not significantly alter the average soil temperatures; no difference was found among different treatments (Figure 3.1a). The average soil volumetric water content, however, was significantly lower ($p < 0.001$) in the active treatment than in the passive and ambient controls in August (Figure 3.1b). In May and June, soil volumetric water content was significantly lower ($p < 0.05$) in active than in ambient controls (Figure 3.1b).

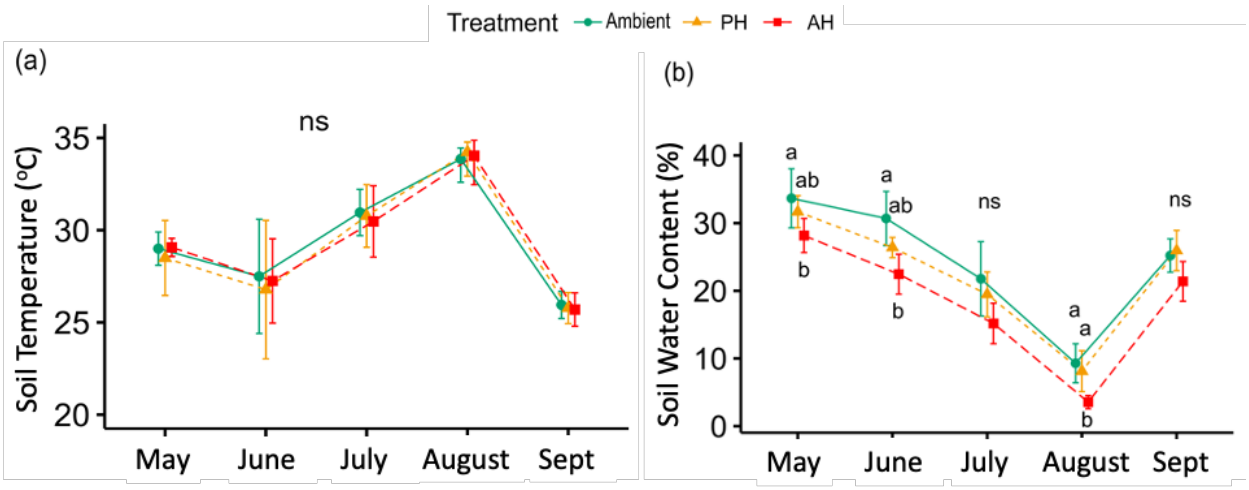


Figure 3.1: Comparison of the average soil temperature (a) and volumetric water content (b) of wild blueberry soils under the active-heating treatment (AH, broken red line with square markers) that consistently raised the mean atmospheric temperature by 3.3 ºC using the heating cables, passive-heating treatment (PH, broken orange line with triangle markers) raised the mean atmospheric temperature by 1.2 ºC compared to the ambient (green line with circle markers) with no chamber throughout the study periods. ns indicates no significant differences among treatments ($p > 0.05$). Dots topped by different letters differ significantly between treatments ($p < 0.01$ in August and $p < 0.05$ for May and June).

3.4.2. Soil Bacterial Community Composition, Abundance, And Diversity

A total of 28,877,870 raw 16S rRNA reads were obtained from 52 soil and control samples under simulated warming treatments and ambient conditions. After trimming and filtering, an average of 301,501 high-quality sequence reads were identified. Comparing each inferred sequence to the others, 38,432 chimeric sequences from 113,120 bacterial sequence variants (SVs) were detected and removed. In this study, using the non-chimeric and rarefied ASVs, we investigated the effect of warming treatments (both the passive and active heating systems) and seasonal changes (late spring, early summer, and late summer) on bacterial richness, Shannon evenness, and diversity. The observed overall richness and Shannon diversity (June, July, and August data combined) of the soil bacterial community under warming treatments (both active and passive heating) did not differ significantly from that of the ambient conditions (Figure 3.2a & b). However, seasonal changes do have a significant effect ($p < 0.05$)

on the observed bacterial richness, Shannon evenness, and diversity, which increased over the growing season (Figure 3.2c & d).

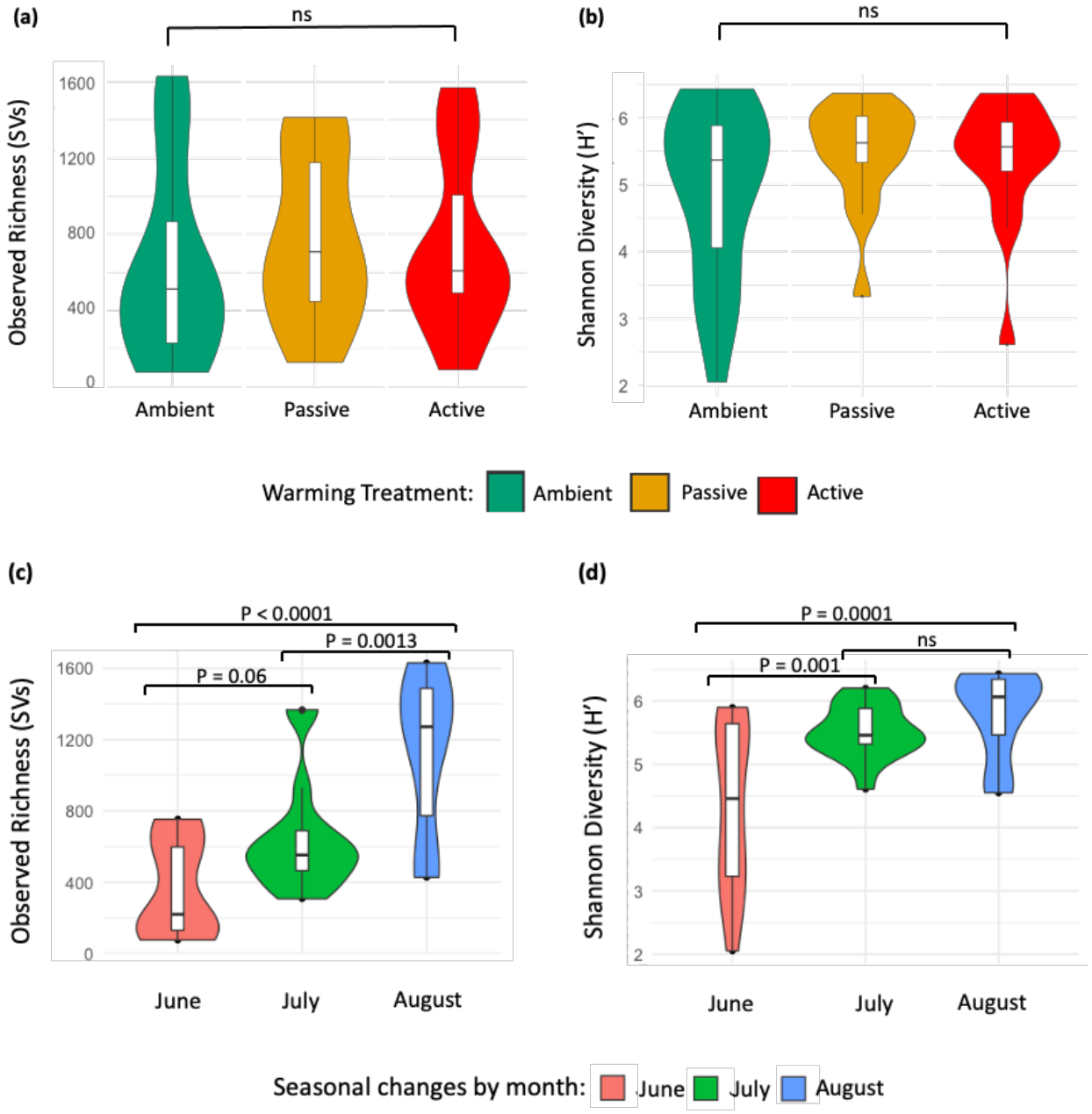


Figure 3.2: Comparison of the overall (across three months) richness (a) and Shannon diversity (b) of wild blueberry soil bacteria communities under warming treatments (passive and active) and the ambient control. There was no significant difference between the warming conditions and the ambient control ($p > 0.05$). Comparison of the (c) Observed richness and (d) Shannon diversity of wild blueberry soil bacteria communities by seasonal differences in late spring (June 09), early summer (July 19), and late summer (August 18) under climate warming treatments. Significant differences existed between the spring and summer seasons under warming conditions compared to the ambient control ($p < 0.05$).

Overall, observed richness, evenness, and diversity (Shannon index) of the bacterial community in the wild blueberry soil increased during the growing season from June to August for all treatments (Figure 3.3). No significant differences were observed in bacterial richness, evenness, and diversity among different treatments in July and August. However, in the spring (June), significantly higher bacterial evenness and Shannon diversity were found in wild blueberry soils under warming treatments (both active and passive), compared to that of the ambient control (Figure 3.3b & c).

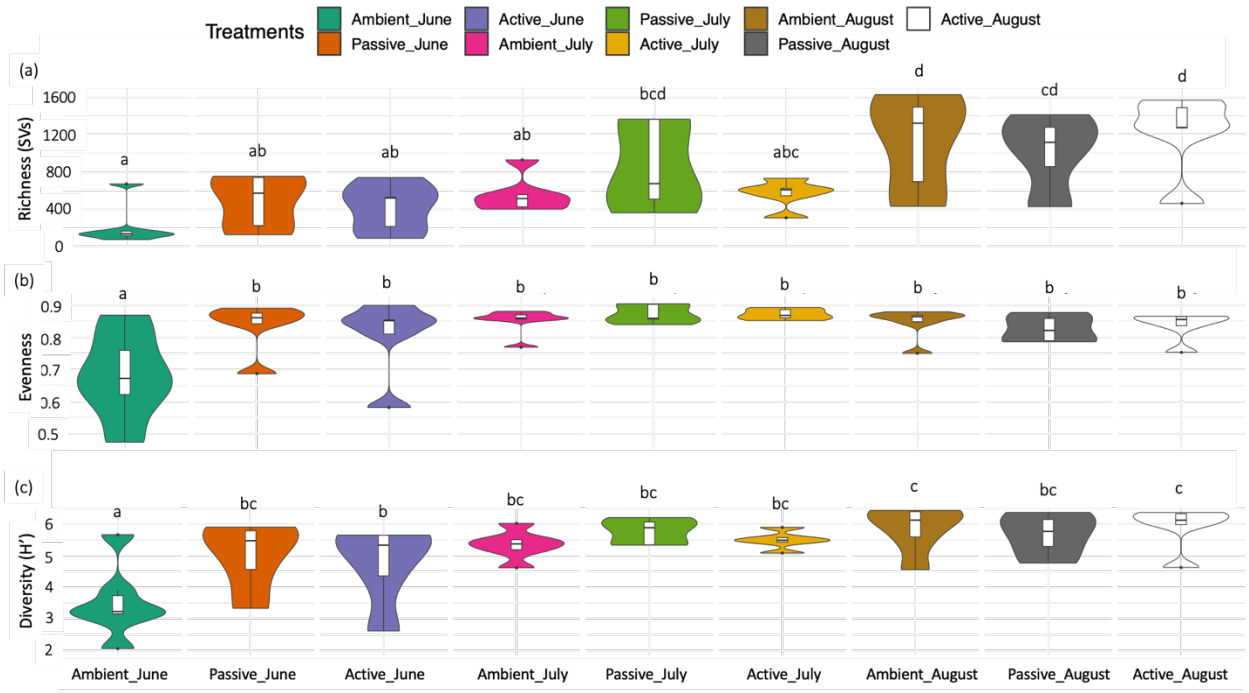


Figure 3.3: (a) Observed richness (SVs), (b) Evenness, and (c) Diversity (Shannon index) for bacteria in the wild blueberry soil under climate warming (Passive and Active) compared to ambient control over two seasons (spring and summer). The thick bar in the middle of the violin box denotes the mean. Letters indicate Fisher's LSD post hoc test following a one-way analysis of variance. All significance levels were tested at an alpha level of 0.05.

Under the elevated temperatures and ambient control treatments, no consistent differences were found in the general soil relative abundance and composition based on the 16S rRNA gene sequence (Figure 3.4). However, as revealed by the data, changes in seasons from

spring to late summer caused the significant effects in the order of phylum and genus in the treatments. At the phylum level, six predominant phyla in order of relative abundance were *Acidobacteriota, Actinobacteriota, Bacteroidota, Planctomycetota, Proteobacteria,* and *Verrucomicrobiota* across temperature treatments and seasons (Figure 3.4). *Firmicutes* and *Bacteroidota* were highly abundant in some samples during the spring compared to the summer. During spring, the elevated temperature has affected some samples' relative abundance of Bacteroidota. The highest relative abundance of ASVs was identified in Acidibacter, which was heterogeneously distributed across different warming treatments. But the lowest relative abundance was found in *Sediminibacterium* at the genus level. Other top-most abundant genera include *Aquisphaera*, *Candidatus Xiphinematobacter*, *Ellin6067* (an unclassified Betaproteobacteria), and *Roseiarcus* (Figure 3.5).

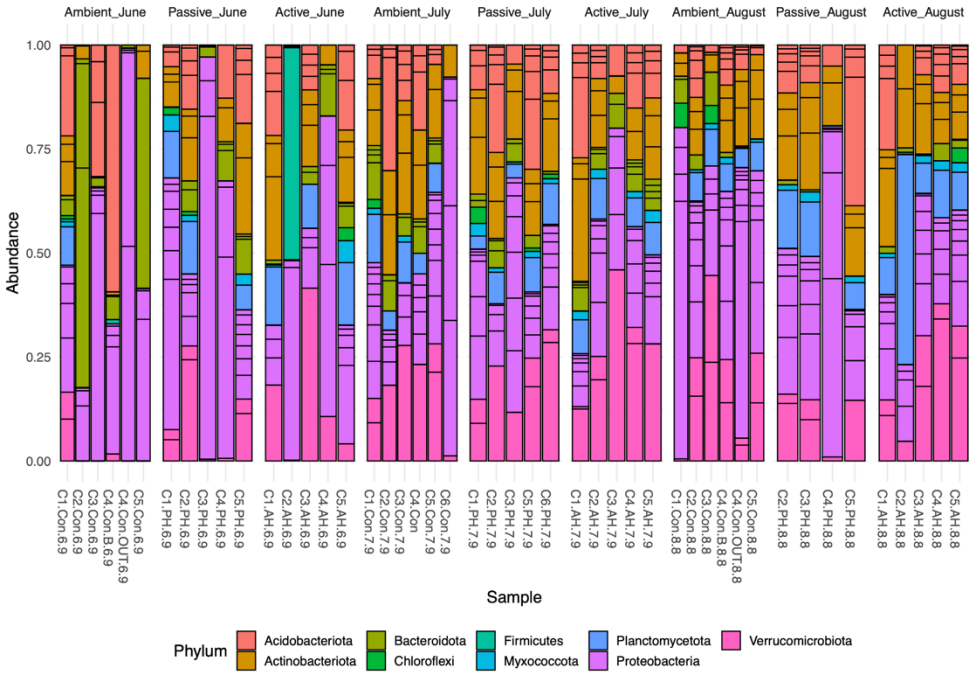


Figure 3.4: Relative abundance at the phylum level under different treatments (ambient, active warming, passive warming) of the wild blueberry soil samples at different times of the growing season (June to August).

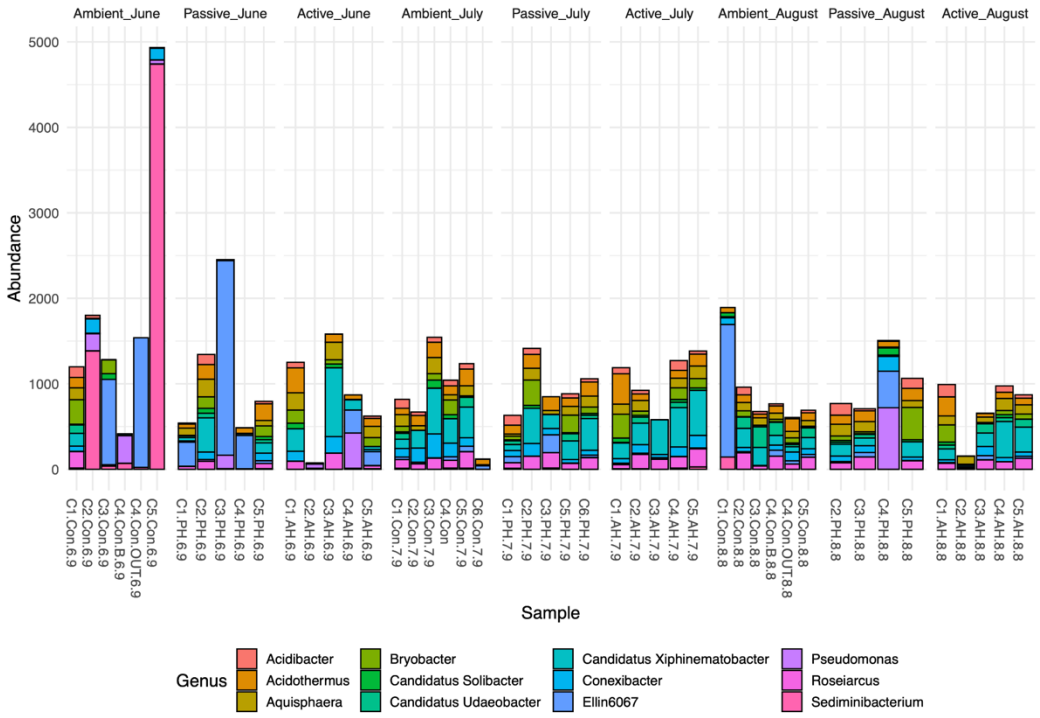


Figure 3.5: Abundance of the topmost 20 bacterial ASVs at the genus levels under different treatments (ambient, active warming, passive warming) of the wild blueberry soil samples at different times of the growing season (June to August).

Even though seasonal changes profoundly caused the taxonomic shift in the genus composition in our study, we plotted the twelve most abundant genera (*Acidothermus, Aquisphaera, Bryobacter, Candidatus Xiphinematobacter, Conexibacter, Ellin6067, Flavobacterium, Methylocapsa, Roseiarcus, Sediminibacterium, Sphingobium, Variovorax*) individually for relative abundance differences between warming treatments and the ambient control. The relative abundance of *Acidothermus* and *Roseiarcus* was lower in the ambient control compared to warming chambers in June (Figure 3.6). *Flavobacterium* and *Sphingobium* showed some outliers in the ambient of June.

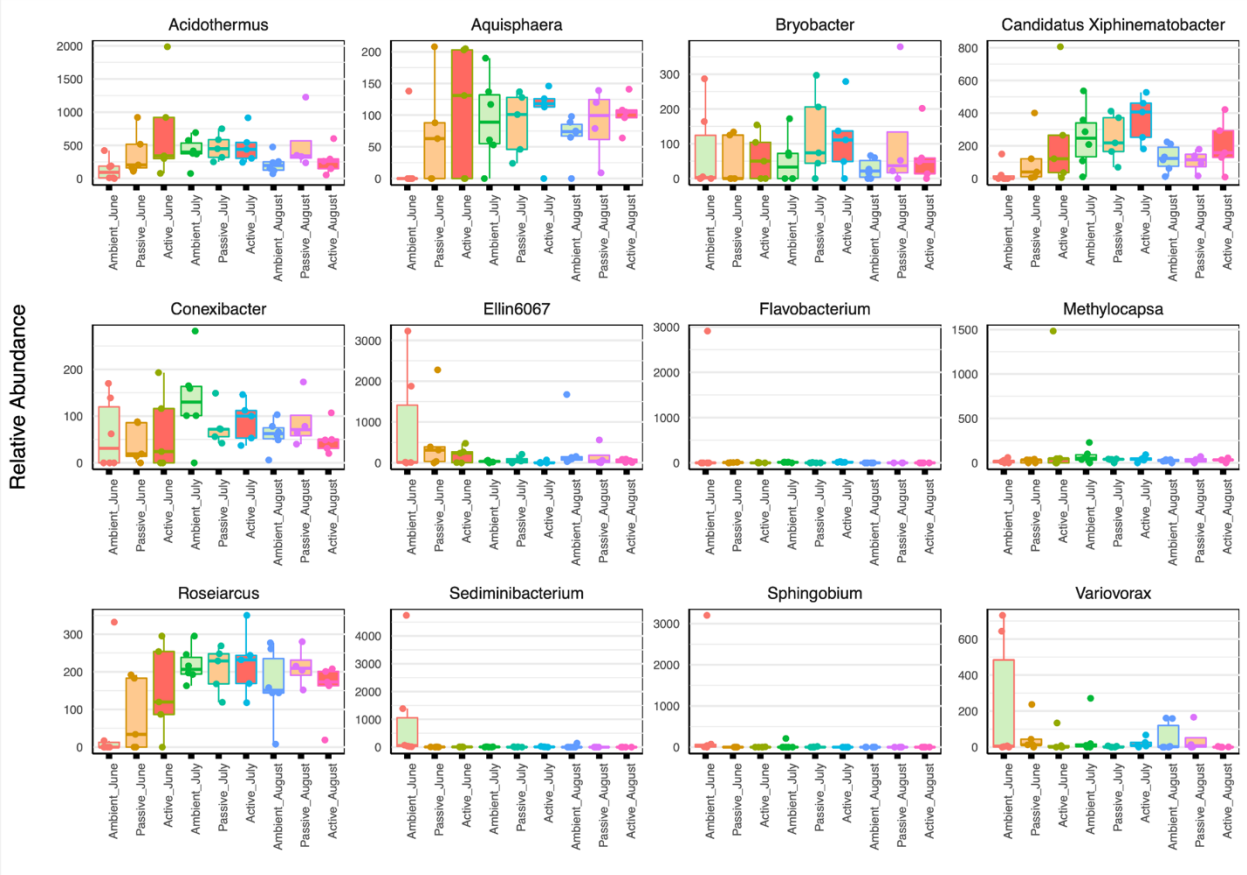


Figure 3.6: Boxplots showing the differences in the relative abundance of wild blueberry soil bacteria at the genus level under warming treatments and as the season changes from spring to summer. Ambient June, Passive June, Active June; Ambient July, Passive July, Active July; Ambient August, Passive August, Active August.

Using a random forest model, we identified the top 30 discriminant genus taxa based on estimated sequence variants with significant importance ($p < 0.05$) under warming treatments as seasons changed from the spring month to summer months (Figure 3.7). The relative abundance of discriminant taxa indicated which bacteria were most likely to be sensitive in wild blueberry farms under elevated temperatures, particularly as seasons change. Members of the genera *Variovorax*, *Acidothermus*, *Bryobacter*, *Candidatus Xiphinematobacter*, and *Aquisphaera* were essential predictors of bacterial abundance in wild blueberry farms under elevated temperatures. Other taxa predictors with relative abundance and importance ($p < 0.05$) include Gemmataceae,

Flavobacterium, and *Acidibacter*. Log relative abundance of Gemmataceae and *Acidibacter* is high in the summer months (mid-growing season) and very low in Spring (early growing season) across the climate warming scenarios. At the same time, *Flavobacterium* showed a contrary pattern (Figure 3.7). The out-of-bag error rate was estimated to be 76.6% in this model, which is too high for the algorithm to accurately differentiate between treatment groups. However, ambient plots in June and July had estimated error rates of 16% and 33%, respectively, indicating that the bacterial communities here were differentiated from all other treatment groups.

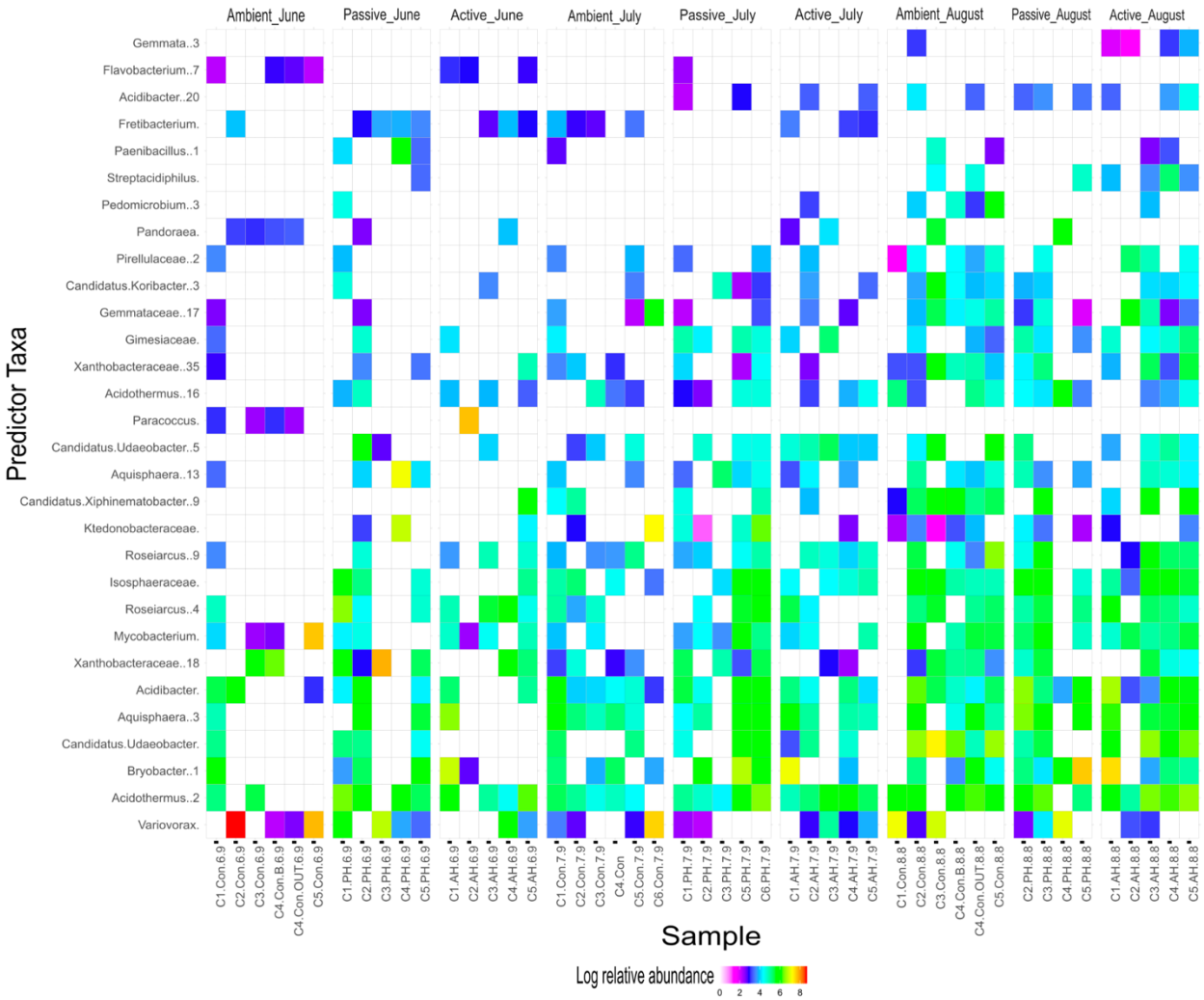


Figure 3.7: Heatmap generated with permutational random forest displaying log relative abundance of top 30 predictor taxa in wild blueberry soils under different heat (ambient, active, and passive) treatments

during climate warming in spring and summer. In white-block samples, discriminant taxa were absent or very low in abundance. The out-of-bag error rate was estimated to be 76.6% in this model.

3.4.3 Effects Of Seasonal Changes And Experimental Climate Warming On Bacterial Community

A Canonical Correspondence Analysis using the Bray-Curtis dissimilarity method was conducted to profile the soil bacterial beta diversity of wild blueberry soils in early and mid-growing seasons (Spring and Summer) using three different temperature treatments. Based on seasonal changes, ASVs clustered significantly but were not significantly affected by open-top chamber warming (Figure 3.8). It appears, however, that raised temperatures using open-top chambers had a more significant effect in the spring than in the summer. Wild blueberry soil bacterial communities differ substantially ($p < 0.05$) comparing the ambient control to the warming treatments (passive and active) in the same late spring season (June). This result may indicate that the effect of warming on soil bacteria communities is pronounced during the early growing seasons of wild blueberry.

However, the soil bacteria communities comparing the warming treatments and ambient control were closely related based on the Bray–Curtis's dissimilarities during the summer season (i.e., July and August) (Figure 3.8). Also, the bacterial community structure in ambient control in late summer (August) and in passive warming treatments in late spring (June) was relatively similar despite different seasons, which may indicate the sensitivity and plasticity of soil bacteria to warming treatment (Figure 3.8).

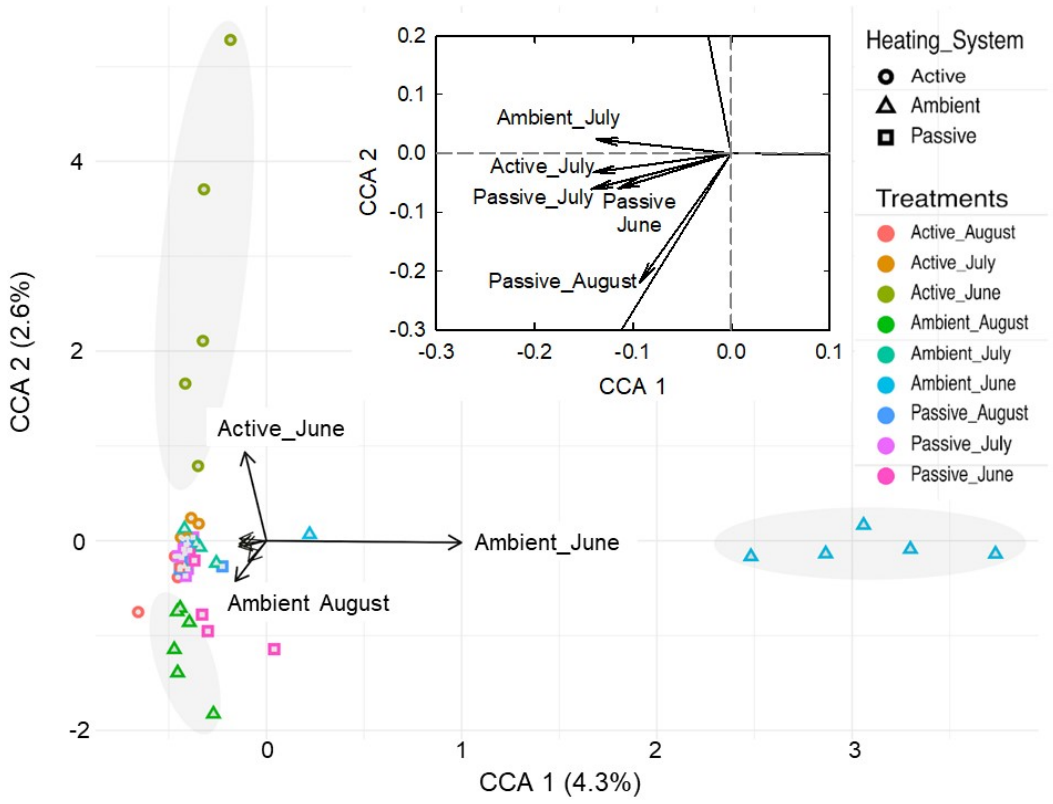


Figure 3.8: Constrained correspondence analysis (CCA) plot showing the dissimilarity in bacterial community composition in wild blueberry soil based on the bray-Curtis method between climate warming treatments and ambient controls in the other months of the growing season. PERMANOVA assessed the significant differences between treatments. The inset shows an enlarged view of the middle part of the figure.

In this study, the Pearson correlation showed that the genus *Acidothermus*, is positively correlated with the genera *Xanthobacteraceae*, *Aquisphaera*, Candidatus *Xiphinematobacter*, and *Roseiarcus*. suggesting that it could interact synergistically with other microbes to promote soil health and increase wild blueberry above-ground biomass. Genus *Sediminibacterium,* which is a prevalent group under ambient temperature in the spring season, is significantly negatively correlated with alpha-diversity parameters (Shannon diversity, observed richness, and evenness) and other highly abundant genera (Figure 3.9).

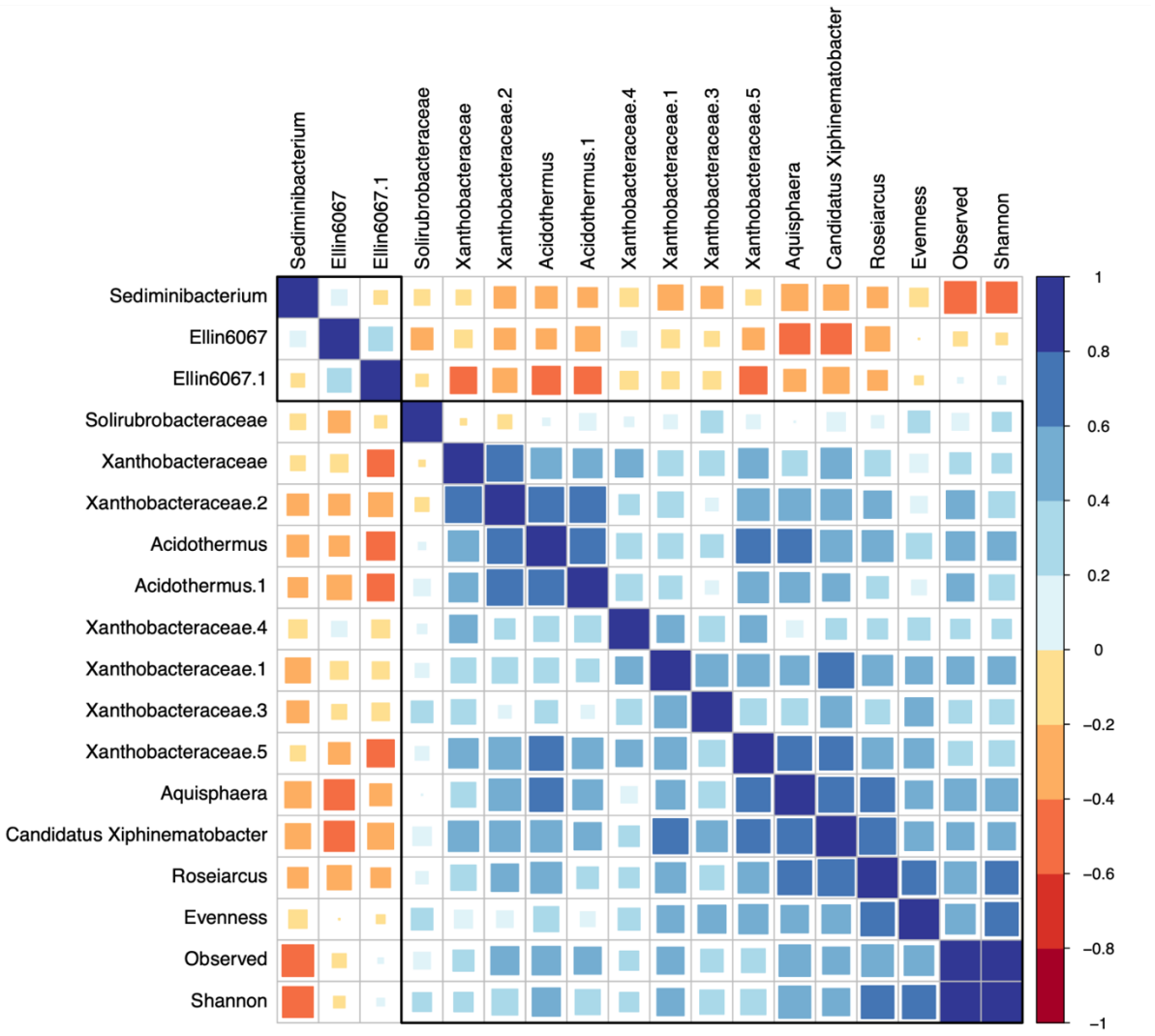


Figure 3.9: Pearson correlations between bacteria communities and alpha diversity metrics. Tiles with white squares represent no correlation, light blue to blue squares denote positive correlations, and orange to red squares denote negative correlations. p-value was adjusted to < 0.05.

The wild blueberry soil bacterial community under warming treatments (both active and passive heating) did not differ significantly from that of the ambient conditions in absolute abundance based on qPCR bacteria copy number (Figure 3.10).

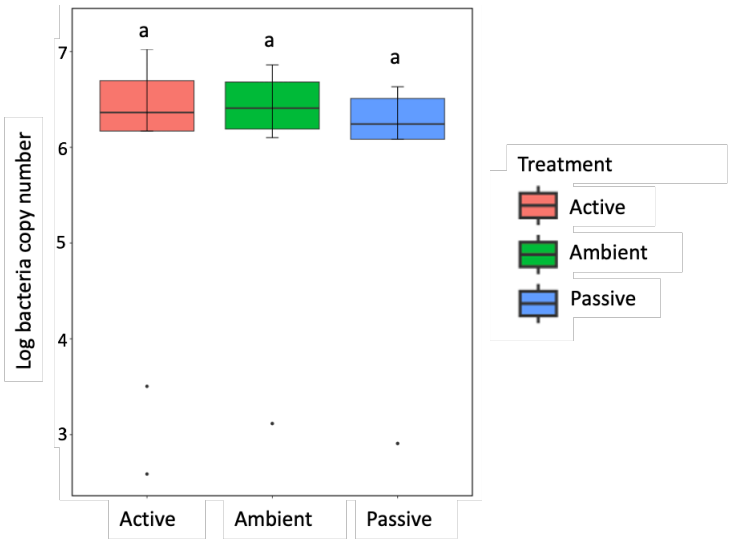


Figure 3.10: Absolute bacteria abundance determined by qPCR analysis of log 10-transformed 16S rRNA gene copies under the different warming treatments: active, passive, and ambient. Box topped with the same letters indicates no significant difference ($p > 0.05$).

The rarefaction curves of bacterial sequence variants in wild blueberry soils under active warming (AH), passive warming (PH), and ambient temperatures (Figure 3.11).

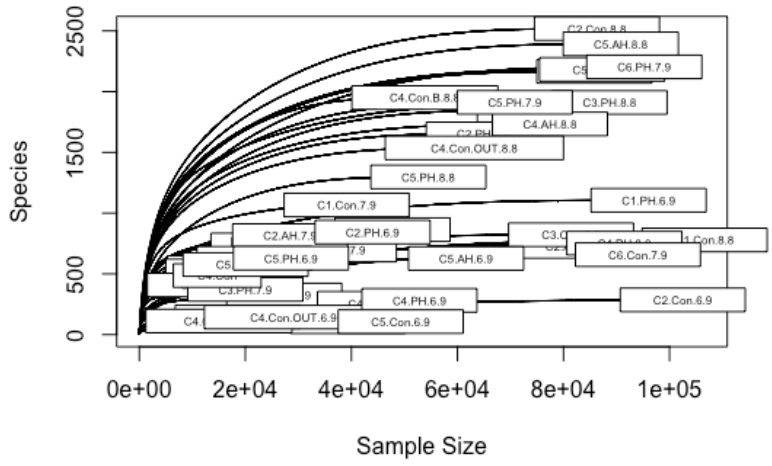


Figure 3.11: The rarefaction curves of bacterial sequence variants in wild blueberry soils under active warming (AH), passive warming (PH), and ambient temperatures.

3.5 Discussion

To the best of our knowledge, this is the first study investigating the bacterial communities of non-amended wild blueberry soils in response to climate warming during the growing season. This study serves as a crucial step in understanding how warming will affect the wild blueberry soil microbiome in the future. Despite an increase in the water deficit in wild blueberry soils caused by continuous warming of 3.3 ^{o}C, there was no significant effect on overall bacterial diversity and richness. Therefore, our hypothesis that warming treatments will drastically alter the diversity and abundance of the bacterial community in wild blueberry soil was not supported. The results from the 16S rRNA phyloseq analysis and qPCR gene copy analyses indicate that the warming of two continuous years did not significantly change the overall abundance, richness, and diversity of the bacteria community (Figures 3.2 and 3.10). This result agrees with other studies (DeAngelis et al., 2015; Schindlbacher et al., 2011; Yang et al., 2022), indicating that only long-term experimental soil warming would cause a significant shift in a microbial community. However, the bacterial evenness and diversity of soils under warming treatment were higher than that of controls in the early growing season (June), probably due to advanced leaf development (Chen et al., 2022). Additionally, we revealed the seasonal dynamics in the wild blueberry soil bacteria community; the bacterial diversity in Summer (July and August) was significantly higher than in late Spring (June) (Figure 3.2c &d). This result is consistent with another study showing that season drives bacteria diversity in tundra soil ecosystems (Pold et al., 2021).

Soil Bacteria Diversity

The soil bacterial diversity under the warming treatment was significantly ($p < 0.05$) higher than that of ambient controls in spring (June). At the same time, there is no significant

difference ($p > 0.05$) among different treatments in summer (Figure 3.3c), suggesting that the effect of climate warming on soil bacterial communities' composition may be more pronounced in relatively cold seasons than in warm seasons. Soil bacterial diversity under all treatments showed significant seasonal changes, which agrees with other studies showing that seasons have a more significant effect than short-term experimental warming in soil ecosystems (Pold et al., 2021; Yuan et al., 2023a; Yang et al., 2022). The bacteria showed the same pattern, warming treatments only changed evenness in June. This result suggests that the evenness of a bacterial community is sensitive to warming in the spring. Because soil temperatures did not differ among treatments, the significantly higher bacterial diversity and evenness is probably due to advanced leaf development (Chen et al., 2022). Earlier leaf development is associated with the production of photosynthates and earlier fine root development, which will consistently change the soil microbial community. Early growing seasons in late spring have been warming up faster than expected in wild blueberry farms over the last two decades (Tasnim et al., 2022). Our results suggest that climate warming may shift the seasonal dynamics of the soil bacterial community in wild blueberry fields.

Bacterial Community Structure

Bacteria communities perform multiple roles in an agricultural ecosystem, including organic compounds decomposition and nutrient acquisition (Burges, 1967; Yuan et al., 2018; Khan et al., 2019; Shah et al., 2021; Prasad et al., 2021). However, these roles are influenced by pH (Zhalnina et al., 2015), vegetation (Ravit et al., 2006; Hui et al., 2017), heavy metal (Kandeler et al., 1996), and soil water content (Brockett et al., 2012; Schimel, 2018). Reduced soil water content under climate warming can markedly alter the diversity, structure, and function of soil microbial communities in cycling carbon of a particular habitat (Rofner et al.

2017). Our results showed that the warming treatments and ambient control share similar soil bacterial communities, indicating no warming effect. Bacteria communities may adapt to short or moderate-term environmental stress levels, including heat and drought (Terzaghi & O'Hara, 1990; Allison & Martiny, 2008). Soil warming over two decades has been reported to shift the bacteria diversity and structure (DeAngelis et al., 2015), indicating that long-term warming, rather than short-term warming, may cause substantial changes in the physicochemical properties of the soil that cause irreversible changes to the bacterial communities.

At the phylum level, Acidobacteriota, Actinobacteriota, Bacteroidota, Chloroflexi, Firmicutes, Myxococcota, Planctomycetota, Proteobacteria, and Verrucomicrobiota were predominantly represented (Figure 3.4). The taxonomic bacteria composition in this study was closely related to the wild blueberry soil bacteria community structure in a previous study (Yurgel et al., 2018). *Acidobacteriota* is the most prevalent phylum comprising a core genus *Bryobacter* (Kulichevskaya et al., 2010), followed by the heat-loving Actinobacteriota phylum comprising common genera such as *Acidothermus* (Viitamäki et al. 2022) and *Conexibacter* (Monciardini et al., 2003). The Acidobacteria phylum synthesizes polysaccharides and phytohormones that control biogeochemical cycles and promote plant growth (Kalam et al. 2020). The phylum *Actinobacteriota* has been described as displaying adaptive strategies to environmental stresses, including warming-induced drought (Sheik et al., 2011; Hu et al., 2023). *Aquisphaera* belongs to the phylum Planctomycetota (Bondoso et al., 2011), *Candidatus Xiphinematobacter* belongs to the phylum Verrucomicrobiota (Vandekerckhove et al., 2000), and the genus *Roseiarcus* is a fermentative bacterium belonging to the phylum Proteobacteria (Kulichevskaya et al., 2014).

Predictor Taxa

Acidothermus and *Candidatus Xiphinematobacter* are relatively abundant in warming soils than in ambient cold springs. Considering the differential abundance of these relatively abundant bacteria taxa, this result may indicate the biological function of the genus *Acidothermus* as a cellulolytic bacterium to recycle plant biomass in warmer environments more rapidly (Viitamäki et al. 2022). These nutrients may enrich the soil and provide bioavailable nutrients to crops.

Conversely, the genera *Flavobacterium*, *Sediminibacterium*, and *Sphingobium* belong to the phylum Bacteroidota (Qu & Yuan, 2008). They are relatively abundant under the ambient temperature in the spring, where they showed higher relative abundance than other warming conditions during the growing season. The sensitivity of these genera to environmental stress, such as the unprecedented warming recently experienced in the Maine coast region (Tasnim et al., 2021), needs to be evaluated. Sediminibacterium has been linked to carbon uptake during substrate biodegradation (Wilson et al., 2016). In our study, Genus *Sediminibacterium* is a prevalent group under ambient temperature in the spring season (Figure 3.6), indicating their specific role in the soil microbes-wild blueberry rhizomes interaction. The relative abundance of Genus *Sediminibacterium* under ambient temperature in the spring might indicate insufficient carbon uptake by the wild blueberry crops.

Acidothermus, a dominant genus showed a positive correlation with genera *Xanthobacteraceae*, *Aquisphaera*, Candidatus *Xiphinematobacter*, and *Roseiarcus*. suggesting that it could interact synergistically with other microbes to promote soil health and increase wild blueberry above-ground biomass. This synergistic interaction between microbes and plant

rhizosphere mediates biochemical processes that influence the growth and stress tolerance of plants (Qu et al., 2020).

Response of Bacterial Composition to Warming and Seasonal Temperatures

Divergence in the beta diversity of bacteria communities corresponds to the response to changes in bacterial richness and alpha diversity (Rui et al. 2015). According to previous studies, warming affects bacterial richness, and alpha diversity at differing rates during the growing seasons, which agrees with our results (Slaughter et al., 2015; Hu et al., 2023). The composition of bacterial communities in wild blueberry soils during the late spring (June, early growing season) and late summer (August, late growing season) were different under ambient conditions, revealing the substantial effect of seasonal variations in microbial composition (Figure 3.8). Under the warming treatments, the bacterial community composition was not similar to that of the ambient control treatment (Figure 3.8). The effect of warming on bacterial communities in the early growing season may be more significant than in the late seasons, according to our study.

3.6 Conclusion

Using high-throughput gene sequencing technology, we analyzed bacterial community diversity and composition in the wild blueberry soil under three different temperature treatments during the growing season. We revealed distinct seasonal changes in the bacterial community. Warming treatments exert a more considerable impact on bacterial communities in the spring than in the summer of the crop-growing season when the seasonal temperature effect is already in play. The Shannon diversity and evenness under warming treatment were significantly higher than ambient spring conditions. In spring, warming significantly advanced wild blueberry phenology (Chen, 2022), which may consequently impact the soil bacterial community.

Therefore, further warming may also shift the seasonal dynamics in bacterial activity in concert with altered plant phenology. The response of soil fungal communities in wild blueberry fields under warming will need to be investigated in the future. Also, this study did not capture the effect of warming during the fall season, which represents another dramatic seasonal change in phenology and needs to be studied in the future. Overall, our study suggests that warming and changes in growing season length could significantly change the dynamics of soil bacterial communities and their interactions with plants.

CHAPTER 4

GENETIC VARIATION OF TWO WILD BLUEBERRY SPECIES ACROSS FOUR FIELDS IN MAINE USING SINGLE NUCLEOTIDE POLYMORPHIC MARKERS

4.1 Abstract

A thorough knowledge of genetic diversity is essential for crop conservation and management. This study examined the extent of genetic variability in a panel of 165 unique wild lowbush blueberry individuals (135 *Vaccinium angustifolium* individuals and 30 *Vaccinium myrtilloides* individuals) collected from four locations in Maine: Hope (n = 32), Jonesboro (n = 38), Deblois (n = 78), and Fort Kent (n = 17) across 350 km using 6491 single nucleotide polymorphic (SNP) markers developed by quantitatively reduced representation sequencing (OmeSeq). The genetic variance within populations was 99 % and 94% in *V. angustifolium,* and *V. myrtillodes,* respectively. The genetic differentiation among three *V. angustifolium* populations was significantly ($p < 0.006$) low (PhiPT = 0.008). Meanwhile, the two *V. myrtilloides* populations showed moderate genetic differentiation (PhiPT = 0.055). The *V. myrtilloides* individuals were found in the very low admixture zone and are predominantly from Fort Kent, while the *V. angustifolium* were found in the low to high admixture zones. *V. angustifolium* individuals from Hope were largely represented in the high admixture zone. Assessment of genetic composition among unmanaged and managed *V. angustifolium* individuals across three locations (Deblois, Hope, and Jonesboro) in Maine using principal coordinate analysis (PCoA) revealed no distinct genetic distance except for a few Jonesboro individuals that were genetically isolated from other individuals, which is confirmed with relatively high PhiPT ranging from 0.007 - 0.013. The high within-population genetic variance agrees with previously found high variation in functional traits and could contribute to their resilience to climate change. The low or moderate among-

population differences suggest gene flow at km distances. The findings of this study provide insights for measuring genetic variability, population structure, differentiation, and the impact of management, which are important for inferring future crop management strategies under climate change.

4.2 Introduction

The wild blueberry (mainly two species: *Vaccinium angustifolium* Ait. and *Vaccinium myrtilloides* Michx.) belongs to the heath family, Ericaceae (Hicklenton et al. 2000). It is a native North American fruit crop distributed across managed and unmanaged fields in Maine (U.S.) as well as Quebec and the Maritime provinces in Canada. This perennial prostrate shrub grows to a height between 0.1 m and 0.4 m. Specifically, *V. angustifolium,* also known as sweet lowbush blueberry, is an outcrosser, autotetraploid ($2n = 4x = 48$) species (Camp 1945; Hokanson and Hancock 1993; Bell et al. 2008) having smooth, glossy leaves, and shortened internodes stem with colors ranging from tan to red. *V. myrtilloides,* also known as sourtop blueberry, is a diploid ($2n = 2x = 24$) species with a characteristic leaf pubescence (Redpath et al. 2022). In addition, half-high blueberries, a hybrid between *V. angustifolium* and *V. corymbosum* (highbush blueberry), also occur in the region (Strik and Yarborough 2005; Jones et al. 2014). Understanding the genetic variation and diversity among wild blueberries and their relationships with ecophysiological adaptation could help predict their response to climate change, and provide information for the breeding of cultivated highbush blueberries. For instance, freezing resistance has been improved in southern highbush blueberries through interspecific crossing with the wild blueberry (Ballington 2009).

Population adaptation to fast-changing environments is highly related to genetic diversity (Reed and Frankham 2003). The northeastern regions of the United States are warming faster

than other regions, especially Maine, where most wild blueberries are produced (Tasnim et al., 2021; Fernandez et al., 2020). Understanding the extent of genetic variability in a population is considered the bedrock for the effective conservation and utilization of crops that can facilitate and sustain crop productivity in the face of climate change (Bhandari et al. 2017). As previous studies have shown that elevated temperature disrupted the wild blueberry physiology and reduced some of the chemical compositions of wild blueberries (Chen et al., 2022; Barai et al., 2021; Alaba et al., unpublished), there is a crucial need to adopt climate-smart wild blueberry production by effective crop management strategies. Genotypes of wild blueberry exhibit wide phenotypic diversity, such as stem height, berry density, and leaf colors (Coville et al. 1937; Wood and Barker 1963; Barai et al. 2022). However, environmental conditions limit phenotypic traits, and a dearth of knowledge exists on the genetic diversity of wild blueberries using high throughput markers (Bell et al. 2010). Molecular markers have been used in a few studies to estimate the level of diversity of wild blueberry fields in Maine and Nova Scotia (Bell et al. 2012; Debnath 2014; Beers et al. 2019). Assessing genetic diversity with molecular markers reveals germplasm backgrounds, relationships, and strategies to establish, utilize, and manage crop core collections. When wild blueberry species are compared to each other to estimate their relatedness, it is possible to eliminate duplicates and reduce genetic erosion among species.

V. angustifolium genetic heterogeneity and relatedness within and among populations have been quantified using various molecular markers (Bell et al. 2008; 2009; 2010; 2012; Burgher et al. 2002; Rowland et al. 2010). These markers include Random Amplified Polymorphic DNA markers (RAPD) (Burgher et al. 2002), Inter-Simple Sequence Repeat (ISSR) (Debnath 2009), and expressed sequence tags-polymerase chain reaction (EST-PCR) (Rowland et al. 2003a, 2003b; Bell et al. 2008). These studies revealed high diversity in *V. angustifolium*

within confined regions of Maine and the Canadian Maritimes (Bell et al. 2012; Debnath 2014; Beers et al. 2019). However, many of these marker systems are expensive and limited by the time needed to discriminate the exact allelic copy number, especially in tetraploid crops like *V. angustifolium*. Genome-wide single nucleotide polymorphic (SNP) markers have become more widely adopted in assessing crop genetic diversity, including fruit crops such as strawberries (Zurn et al. 2022) and highbush blueberries (Campa and Ferreira 2018). These markers are abundant in the genome, amenable to high throughput genotyping techniques, locus-specific, and have a low sequencing cost per data point. Compared to *V. corymbosum*, *V. angustifolium* has not benefitted from recent advances in high-throughput sequencing technology. OmeSeq is an emerging high-throughput quantitative reduced representation sequencing technology for exploring genetic diversity in complex plant species (Kuster et al. 2021). Additionally, the genetic diversity and structure of *V. myrtilloides* have not been studied.

The genetic diversity of wild blueberries could be influenced by both management and natural seed dispersal. Commercial wild blueberry fields are usually established by clearcutting forests, which expose the natural understory wild blueberry plants directly to sunlight and allow the underground stems to spread (Morrison et al. 2000; Abdalla, 1967) and the stems to produce flowers and, subsequently, fruit. The monoecious floral organ of wild blueberry is pollinated mainly by bumble bees, digger bees, plasterer bees, and sweat bees (Bushmann and Drummond 2015). Other agents of pollination, including moths and hummingbirds, have been observed in wild blueberry fields. Natural seed dispersal is through animal-mediated frugivory, especially by migratory birds and bears (Asare et al. 2017; Drummond 2019). Seeds germinate at temperatures between 16 and 21 °C after one month of dormancy. Although seedlings, softwood cuttings, and micropropagation have been explored for wild blueberry propagation (Bell et al.,

2009), only naturally-growing plants are used for commercial production. In an attempt to select *V. angustifolium* genotypes for high yield, Hall (1983) found a significant increase in mean berry size from progenies of selected genotypes compared to open-pollinated parents. Thus, the genotypic differences can be harnessed to increase productivity in wild blueberries, and robust and advanced molecular tools can be used to understand the extent of genetic variation and to map important heritable traits. However, wild blueberry has a lengthy progeny establishment period, high heterozygosity, and lacks robust molecular marker systems that are useful in understanding this unique system (Bell et al. 2009; Rowland et al. 2010). Although a breeding program is not feasible for the natural wild blueberry production system, understanding their genetic diversity and structure could help inform its management under climate change and breeding resilient highbush blueberry varieties.

To this end, this study aimed to adopt SNP markers for the first time to assess the genetic diversity and structure level in 165 blueberry individuals belonging to two wild blueberry species (*V. angustifolium* and *V. myrtilloides)* across a geographic range of 350 km in Maine. Seven varieties of commercial highbush blueberry (*V. corymbosum*) were also included to show their relatedness. This study will lay the foundation for identifying genetic relatedness in wild blueberry genotypes. Objectives of this study were to: 1) Assess the genetic diversity and differentiation in three populations of *V. angustifolium* and three populations *of V. myrtilloides* using SNP marker data; 2) Test whether populations geographically distinct could be separated from each other by marker profiles; and 3) Test whether plants collected in managed commercial fields show distinct molecular marker profiles compared to plants collected in neighboring non-managed habitats.

4.3 Materials And Methods

4.3.1 Plant Material

Leaves of a total of 165 wild blueberry plants of two species were collected from four geographically distinct locations in Maine: Hope Farm (44.1750°N, 69.1113°W) in Hope, Knox County, Blueberry Hill Research Farm (BBHF; 44.3600°N, 67.3600°W) in Jonesboro, Washington County, Wyman's Farm (44.4200°N, 68.0000°W) in Deblois, Washington County and Fort Kent (47.2185°N, 68.5982°W) in Aroostook County (Figure 4.1 and Table 4.1). These sites were selected to cover the range of wild blueberry fields in Maine, spanning 350 km. Samples were kept in sealed plastic bags and transported to the University of Maine pathology laboratory for DNA extraction. Specifically, 30 individuals of *V. myrtilloides* were randomly collected from Fort Kent, Jonesboro, and Hope. A total of 135 *V. angustifolium* individuals were collected from Deblois, Hope, and Jonesboro. While only *V. myrtilloides* was found in the fields at Fort Kent, only *V. angustifolium* was found in the field at Deblois. Some of the *V. angustifolium* individuals were collected in the unmanaged forest (woods) adjacent to the managed fields. Among these 135 *V. angustifolium* individuals, 31 were replicated five times (North, South, Center, West, and East regions) from a morphologically unique genotype and are referred to as transplant parents (TPs). To validate the fidelity of the genotyping platform, we triplicate analyzed one sample (S7).

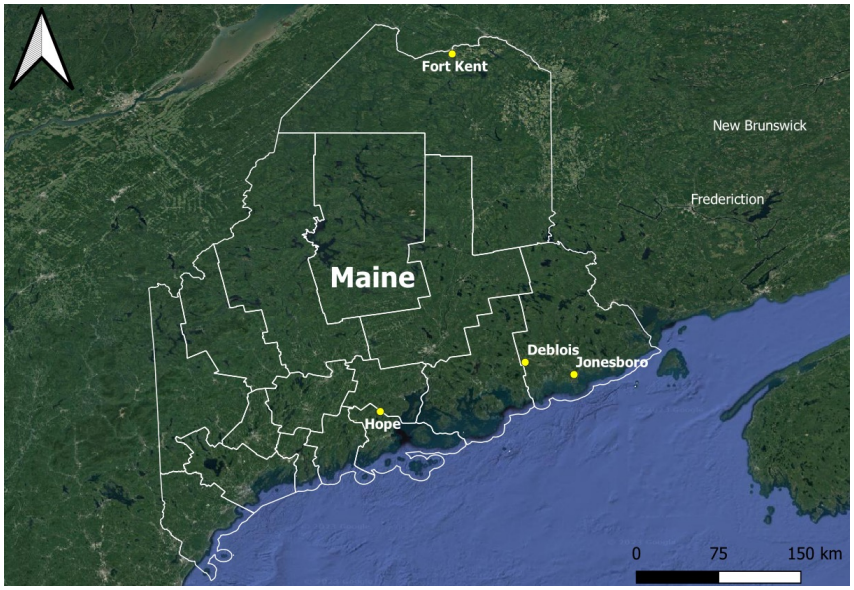


Figure 4.1: A map showing sampling locations (represented by yellow dots) of two wild blueberry species (*Vaccinium angustifolium* and *V. myrtilloides*) across Maine. Map created with QGIS (QGIS Development Team 2009).

Table 4.1: *Vaccinium angustifolium* and *Vaccinium myrtilloides* sample size and geographic distribution.

Species	Geographical Locations	Individual size (n)
V. myrtillodes	Fort Kent	17
	Hope	2
	Jonesboro	11
V. angustifolium	Deblois	78
	Hope	30
	Jonesboro	27

4.3.2 DNA Isolation

The total genomic DNA from 50 mg of fresh young leaf tissue collected from each sampled plant was isolated using CTAB (cetyltrimethylammonium bromide) extraction methods with slight modifications (Doyle and Doyle 1987). Briefly, wild blueberry leaf tissues were punched into labeled 2-mL Eppendorf tubes containing three steel balls and 400 µL of CTAB Buffer with β-Mercaptoethanol and RNase A mixture. The 2-mL Eppendorf tubes containing submerged leaf tissues were capped and ground in FastPrep-24 homogenizer (MP Biomedicals, Santa Ana, CA, USA) at 30hz for 2 min. Using a water bath at 65 °C, samples were incubated for 15 min with intermittent vortexing. After incubation, 400 µL of chloroform: isoamyl alcohol (24:1) was added to the samples, gently vortexed, and centrifuged at 10,000 rpm for 10 min. The top aqueous layer from samples (400 µL) was transferred to newly labeled tubes using a pipette. Two hundred and seventy-six µL of isopropanol was added to each tube, mixed gently by inversion, and kept overnight at -20 °C. After that, samples were centrifuged at 12,000 rpm for 10 min, then the supernatant was carefully discarded, and DNA pellets were washed twice by adding 200 µl of 70% ethanol. The tubes containing DNA pellets were kept on Kim wipes in the flow hood until the 70% ethanol evaporated. Finally, the dried DNA pellets were 50 µl of autoclaved low-salt TE buffer. The DNA concentration (ng/uL) was determined using the NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific Inc., Denver, CO, USA) at an absorbance of 260 nm. Using gel electrophoresis, DNA quality was assessed from randomly selected samples in 1 % agarose gel stained with SYBR Safe, and the images were viewed under ultraviolet light.

4.3.3 Quantitative Reduced Representation Sequencing (Omeseq-Qrrs) Library Preparation

DNA samples were quantified (Quant-iT PicoGreen dsDNA assay, Invitrogen, Waltham MA, USA), and normalized to 20 ng/uL before the OmeSeq-qRRS library preparation method. The library preparation strategy represents a small fraction of the genomes by performing a double digest with NsiI-HF and NlaIII restriction enzymes (RE) and selecting only 300-600 bp fragments flanked by both RE motifs. Using isothermal amplification and the Bst2.0 WarmStart DNA Polymerase (NEB) strand displacement activity, modified Illumina P5 and P7 single-stranded and barcoded adapters were incorporated into the genomic fragment ends at the NsiI-HF and NlaIII overhangs, respectively. The 96 sets of the P5 and 96 P7 adapters allowed for multiplexing up to 9, 216 samples. The modified adapters contained 6 bp buffer sequences, 7-10 bp long variable length barcode, and 4 bases complementary to the RE overhangs. OmeSeq-qRRS provided a quantitative assay by mitigating shortcomings of ligation-based and multiplexed PCR-based methods by eliminating off-target amplification and chimeric reads. The library preparation entailed two multi-step reactions. The first multi-step reaction entailed DNA nick repair, digestion, and incorporation of 96 single-stranded P5 custom adapters based on isothermal amplification. The DNA nick repair ensured that partially degraded samples were well represented. The thermal cycler conditions were 37 °C for 30 minutes (phosphorylation with T4 polynucleotide kinase), 16 °C or room temperature for 30 minutes (nick repair with E. coli ligase), 37 °C for 3 h (digestion with NsiI-HF), 65°C for 10 mins (isothermal amplification with Bst 2.0 WarmStart DNA polymerase, while other enzymes were heat-killed), cooled at 2 °C/min until a temperature of 20 °C was reached. The samples were then held at 4 °C. The second multi-step reaction comprised restriction digest and isothermal amplification

incorporating the P7 single-stranded adapters. The thermal cycler conditions were 37 °C for 3 h (digestion with NlaII), and 65 °C for 30 min (isothermal amplification with Bst 2.0 WarmStart DNA polymerase, while the NlaIII enzyme is heat-killed). The samples were then cooled at 2 °C/min until they reached 20 °C, after which they were held at 4 °C. Equal volumes of all the samples were pooled into a single tube, purified, and size selected with 1.2X Pronex mag bead (Promega, Madison, WI, USA) to exclude adapter dimers. Additional size selection (300 - 600 bp DNA fragments) was performed using the BluePippin™ electrophoresis equipment (Sage Science™, MA, USA) and a 2% Agarose Gel Cassette (Sage Science™). The size selected DNA library was qPCR enriched at 18 cycles (NEB Q5 high-fidelity DNA polymerase) and mag bead purified with 1.2X Pronex mag bead. The library was pair-end sequenced (2 x 151 bp) on the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA).

4.3.4 Demultiplexing, Quality Filtering, Variant Calling, And Variant Filtering

The Illumina short reads were demultiplexed and quality-filtered with ngsComposer, a completely automated and user-friendly pipeline (Kuster et al. 2021; https://github.com/bodeolukolu/ngsComposer). The workflow included the trimming of the 6 bp buffer sequence, demultiplexing reads with a maximum mismatch of 1, removal of reads without the entire restriction enzyme recognition site, end-trimming of low-quality bases with PHRED quality score threshold of 30, adapter detection and removal, and elimination of reads that had more than 20% of bases with PHRED quality scores lower than 30. The variant calling and filtering was performed with GBSapp (https://github.com/bodeolukolu/GBSapp; Kuster et al. 2021). This automated and user-friendly software uses original and third-party bioinformatic tools and implements several best practices before, during, and after variant calling. Dosage-

sensitive variant calling was performed with Genome Analysis Toolkit v4.2.6.1 (McKenna et al. 2010).

4.3.5 Data Analysis

A genetic distance matrix was developed to construct a weighted neighbor-joining (NJ) dendrogram in DARwin software version 6.0.021 (Perrier and Jacquemoud-Collet, 2006) with 1000 bootstrapping. STRUCTURE 2.2 (Pritchard et al. 2000) software was used to infer the likely population structure and classify admixed wild blueberry individuals with a 10,000 burn-in period, followed by 50,000 iterations. Allele frequencies were used at each independent SNP locus to identify the number of subpopulations (K) likely to exist (Evanno et al. 2005). At each subpopulation (K) level, with assumed K ranging from one to nine, 20 independent runs were conducted, thereby clustering individual samples based on genotype. With the software GenAlEx (Peakall and Smouse 2006), we computed an analysis of molecular variance (AMOVA) with 9999 permutations for partitioning the genetic variations within- and among populations across different field locations in Maine.

4.4 Results

4.4.1 Genome Sequencing And SNP Calling

Wild blueberry sequencing using the quantitative reduced representation (OmeSeq) resulted in a total of 187,216,012 raw reads after demultiplexing and quality filtering. Of these raw reads, 93.67% mapped to the reference genome (*Vaccinium darrowii* Camp), a wild diploid blueberry, referred to as Southern Highbush blueberry. These reads were used for variant calling to generate a total of 551,755 SNPs subjected to SNP filtering. The SNP filtering was based on a minor allele frequency (MAF) threshold of 0.02, a sample missingness threshold of < 0.3, and an SNP missingness threshold of 0.3. A read depth threshold 4 was used and evaluated on each

genotype call rather than the average read depth for each variant. Genotype calls below this read depth threshold were re-coded as missing genotype data. After SNP filtering, a total of 7,407 quality SNPs were retained across 271 samples, which were used in the downstream analysis. We added 2 *V. myrtilloides* of known origin (Hope and Jonesboro) as controls. Transplant parents (TPs) in the *V. angustifolium* group were used as biological replicates. The technical replications (S7) were produced in triplicate to validate the fidelity of the genotyping platform. As expected, the replicates of a genotype grouped together, confirming their identical genetic composition (Figure 4.5).

A total of 7,235 SNPs (97.7% of total SNPs) were mapped to the 12 chromosomes of the blueberry reference genome (*V. darrowii*), while the remaining 172 SNPs were mapped to the unassembled scaffolds and contigs. The SNPs were evenly distributed across the genome (Figure 4.2).

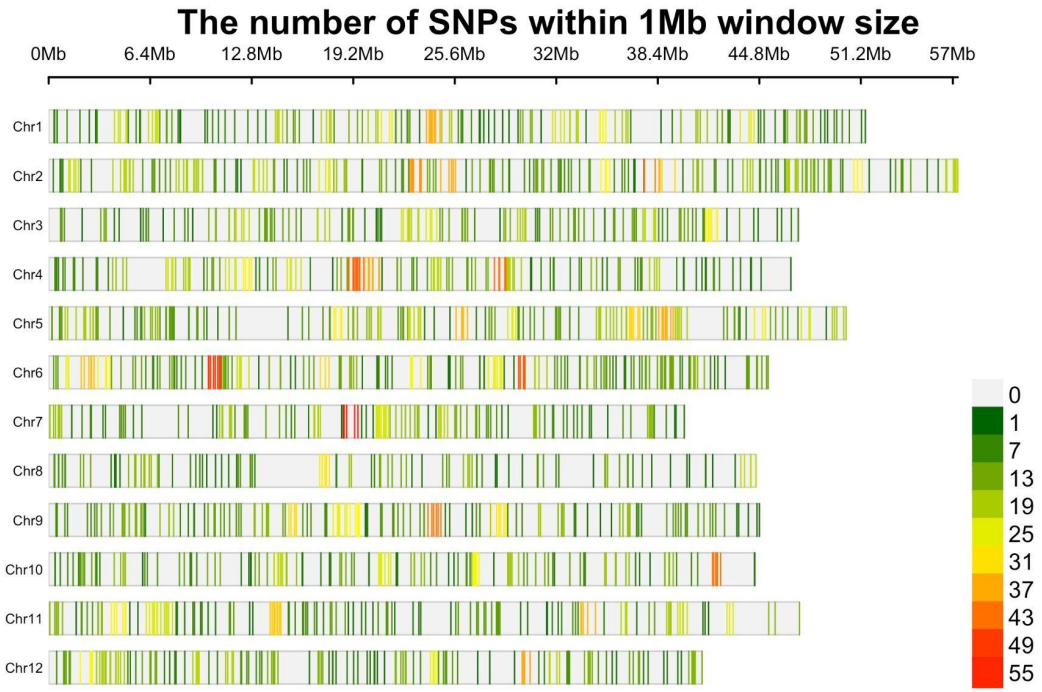


Figure 4.2: SNP markers distribution across the reference genome (*V. darrowii*) within a 1 Mb window size. The marker density is represented by different bar colors.

4.4.2 Genetic Variance Of *Vaccinium angustifolium* Population Collected In Different Regions

Using the high-quality SNPs to determine the source of molecular variance (AMOVA) within and among the population. A 99 % source of variation was found to be within *V. angustifolium* populations (Deblois, Hope, Jonesboro), while variation among populations was 1% (Table 4.2).

Table 4.2: Analysis of molecular variance (AMOVA) in *V. angustifolium* only across three locations (Deblois, Hope, Jonesboro) with 9,999 permutations. In our data, 99% of the genetic variance is explained within populations, and 1% is explained among populations.

Source	**df**	**SS**	**MS**	**Est. Var.**	**%**
Among Pops	2	2787.989	1393.995	8.935	1%
Within Pops	132	138088.099	1046.122	1046.122	99%
Total	134	140876.089		1055.057	100%

Stat	Value	*p*
PhiPT	0.008	0.006

The overall PhiPT (Fst analog from Wright's F statistics, (Wright 1965; Peakall and Smouse 2012) for *Vaccinium angustifolium* among the three sampling locations, estimated using 6491 SNPs, was low (0.008) but significant ($p < 0.006$). The range of values of PhiPT that indicate pairwise population differentiation from each location varied from 0 to 0.013, with the Jonesboro population distantly related to Hope (PhiPT = 0.013) (Table 4.3).

Table 4.3: Pairwise genetic differentiation (PhiPT) among *V. angustifolium* across sampling locations based on 6419 SNP markers.

	Deblois	Jonesboro	Hope
Deblois	0.000		
Jonesboro	0.007	0.000	
Hope	0.008	0.013	0.000

We further assessed the genetic composition of *V. angustifolium* populations using the principal coordinate analysis. There appears to be no distinct genetic differentiation among *V. angustifolium* populations assessed (Figure 4.3).

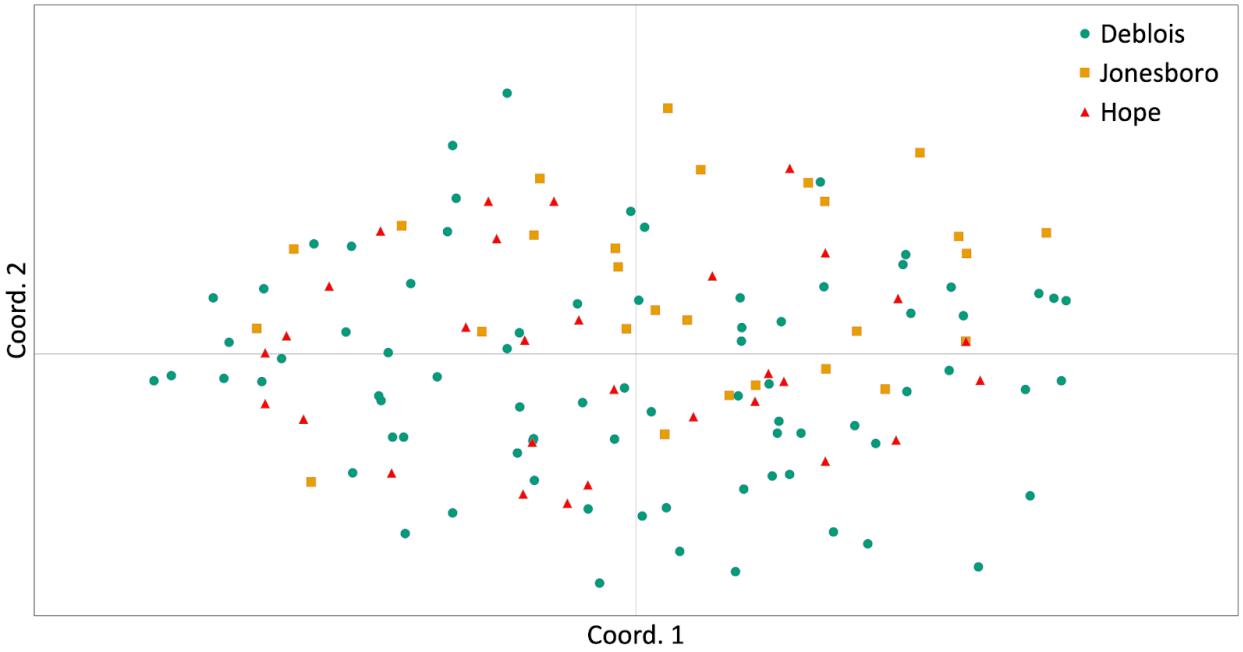


Figure 4.3: Two-dimension plot generated from principal coordinate (PCoA) analysis for *V. angustifolium* from three locations (Deblois, Jonesboro, and Hope) in Maine using 6491 SNP markers.

4.4.3 Genetic Variation Of *Vaccinium myrtilloides* Population Collected In Different Regions

We assessed the genetic variation and composition among *V. myrtilloides* across geographical locations in Maine also using AMOVA and principal coordinate analysis. Using 6491 SNPs, the genetic variance observed within *V. myrtilloides* populations was 94% with a relatively high PhiPT (0.055) ($p < 0.000$) (Table 4.4). Based on PhiPT (0.055), Fort Kent and Jonesboro populations showed moderate genetic differentiation (Table 4.5). The PCoA showed a

distinct genetic differentiation of the Fort Kent population from the population in Jonesboro.

(Figure 4.4).

Table 4.4: Analysis of molecular variance (AMOVA) in *V. myrtilloides* across two locations (Fort Kent, Jonesboro) with 9,999 permutations. In our data, 94% of the genetic variance can be explained within the population, and 6 % can be explained among the populations.

Source	**df**	**SS**	**MS**	**Est. Var.**	**%**
Among	1	1448.116	1448.116	47.492	6%
Within	26	21157.706	813.758	813.758	94%
Total	27	22605.821		861.250	100%

Stat	Value	*p*
PhiPT	0.055	0.000

Table 4.5: Pairwise genetic differentiation (PhiPT) among *V. myrtilloides* across sampling locations based on 6419 SNP markers.

	Jonesboro	Fort Kent
Jonesboro	0.000	0.000
Fort Kent	0.055	0.000

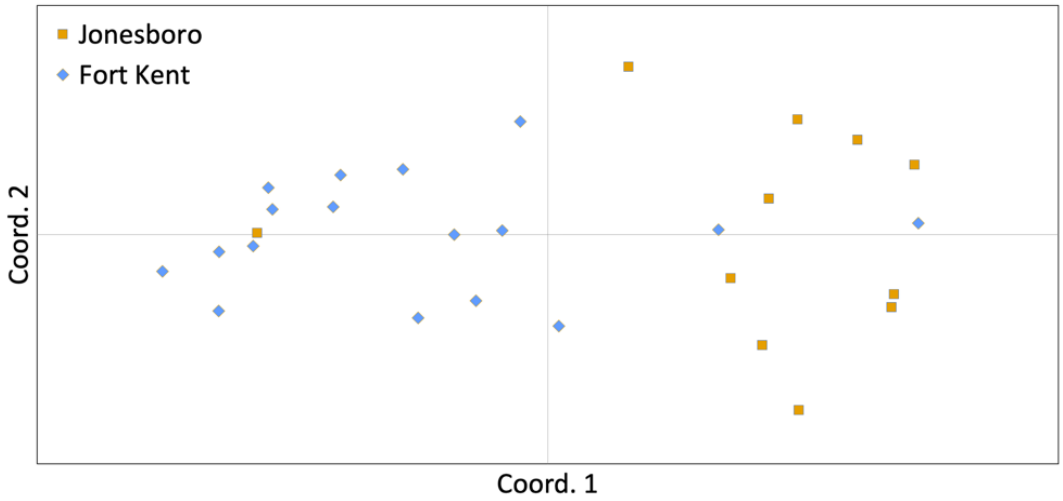


Figure 4.4: Two-dimension plot generated from principal coordinate analysis for *V. myrtilloides* from three locations (Fort Kent, Hope, and Jonesboro) in Maine using 6491 SNP markers.

4.4.4 Overall Genetic Diversity And Structure

The genetic relationships based on the dissimilarity matrix among the Vaccinium samples (n = 165, after dropping the duplicates) collected in three Maine counties were displayed using a neighbor-joining tree (Figure 4.5). There is a main divergence observed between the *V. myrtilloides* (diploid species) and the *V. angustifolium* (tetraploid species) clades (Figure 4.5). Most of the samples in the *V. myrtilloides* group were collected from both managed and unmanaged fields and had a high bootstrap value. Although no samples from Deblois' unmanaged field were found in the Fort Kent clade (Figure 4.5), there was no grouping in this study according to region or location. *Vaccinium corymbosum* (Northern Highbush Blueberry) appeared to be nested within the *V. angustifolium* group. This result was also supported by our genetic structure analysis (Figure 4.6b)

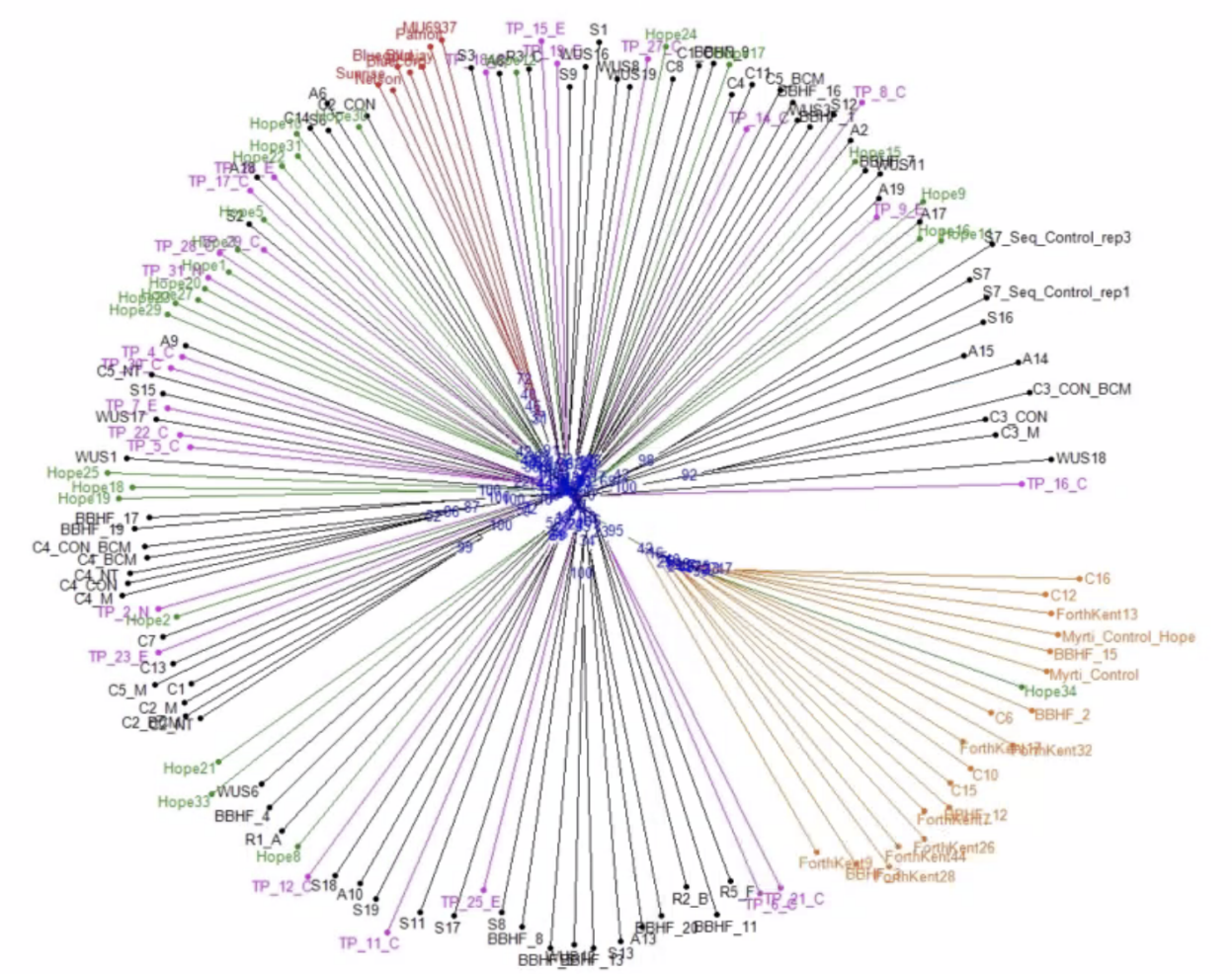


Figure 4.5: Neighbor-joining dendrogram of 165 unique *Vaccinium spp*. genotypes based on the analysis of 6491 SNPs obtained by OmeSeq. The purple color indicates transplant parents (TP) genotypes from the Deblois Town in Washington County. The green color indicates genotypes from Hope Town in Knox County. The orange color indicates genotypes from Fort Kent in Aroostook County. Genotypes labeled as BBHF are samples from Jonesboro**.**

The optimal subpopulation size is estimated by Delta K using STRUCTURE Harvester (Figure 4.6a). The Delta K plateaus at a subpopulation size (K) of 4, the sub-optimal population size. The genetic structure of the *Vaccinium* species collected in three Maine Counties (Aroostook, Washington, and Knox) showed four subpopulations (K = 4) (Figure 4.6b). The four clusters are the *V. myrtilloides* (very low admixture zone) and three distinct sub-groups of the *V.*

angustifolium (low, moderate, and high admixture zones). The high admixture zone comprises admixture individuals with significant allele sharing across all *V. angustifolium* populations. The low admixture zone is largely dominated by *V. angustifolium (*TPs individual) with some *Vaccinium corymbosum* individuals. *V. angustifolium* individuals from the Hope are largely represented in the high admixture zone (Figure 4.6b). The *V. myrtilloides* population showed the lowest admixture proportion and was predominantly from the Fort Kent geographical location.

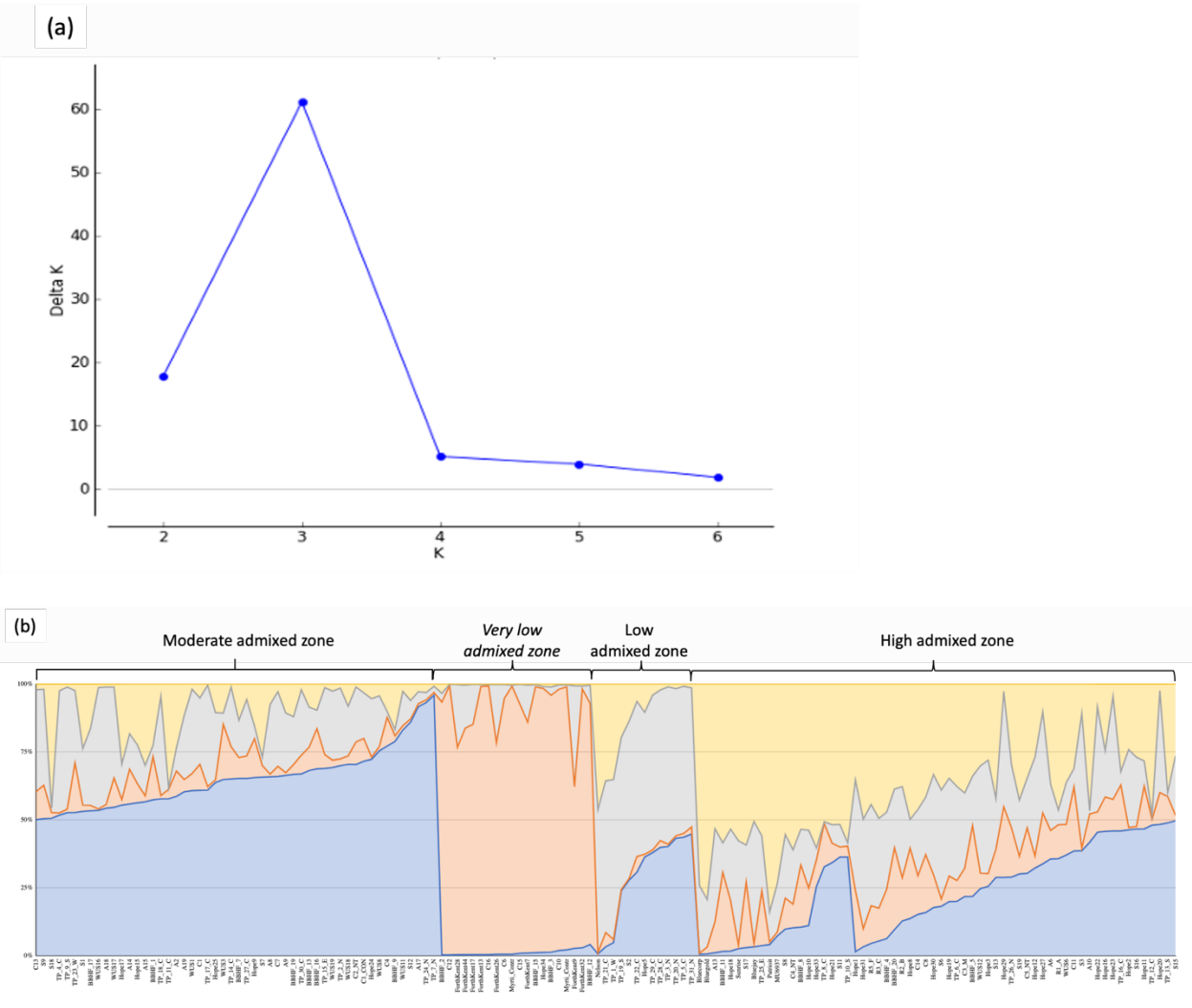


Figure 4.6: (a) The plot of Delta K against the likelihood of K (subpopulations) is described by Evanno et al. (2005) and calculated as the mean of the second-order rate of change. (b) Inferred clusters (K = 4) using STRUCTURE were based on the 6491 SNP markers for the 165 unique individuals sampled across all locations. The x-axis is the samples. The y-axis shows the coefficient of assignment to a population.

Individuals with multiple colors have admixed genetic backgrounds. Each color indicates the most likely ancestry of the cluster from which the genotype shared alleles or derived.

4.4.5 Genomic Variation Of Wild Blueberries In Managed And Unmanaged *V. angustifolium* Populations Across Three Different Locations

Using the SNPs to determine the source of molecular variance (AMOVA) within and among the managed and unmanaged population. A 99 % genetic variance was found within the populations. The PhiPT was 0.008 for these populations ($p < 0.006$) (Table 4.6).

Table 4.6: Analysis of molecular variance (AMOVA) across three locations (Deblois, Hope, Jonesboro) for managed and unmanaged fields with 9,999 permutations. In our data, 99% of the genetic variance can be explained within the population, and 1% can be explained among the populations.

Source	df	SS	MS	Est. Var.	%
Among	5	6045.984	1209.197	8.773	1%
Within	131	136701.899	1043.526	1043.526	99%
Total	136	142747.883		1052.299	100%

Stat	Value	*p*
PhiPT	0.008	0.006

The range of values of PhiPT that indicate pairwise genetic differentiation among populations varies from 0.00 to 0.019, with the Jonesboro unmanaged and Hope managed populations most genetically distant (Table 4.7).

Table 4.7: Pairwise comparison using AMOVA with 9999 permutations across three locations for managed and unmanaged fields.

	Deblois Managed	Deblois Unmanaged	Jonesboro Unmanaged	Jonesboro Managed	Hope Managed	Hope Unmanaged
Deblois Managed	0.000					
Deblois Unmanaged	0.001	0.000				
Jonesboro Unmanaged	0.012	0.006	0.000			
Jonesboro Managed	0.009	0.008	0.002	0.000		
Hope Managed	0.009	0.011	0.019	0.015	0.000	
Hope Unmanaged	0.006	0.008	0.015	0.011	0.002	0.000

We further assessed the genetic composition of managed and unmanaged wild blueberry populations using the principal coordinate analysis. There appears to be no distinct genetic distance among these managed and unmanaged wild blueberry populations (Figure 4.7).

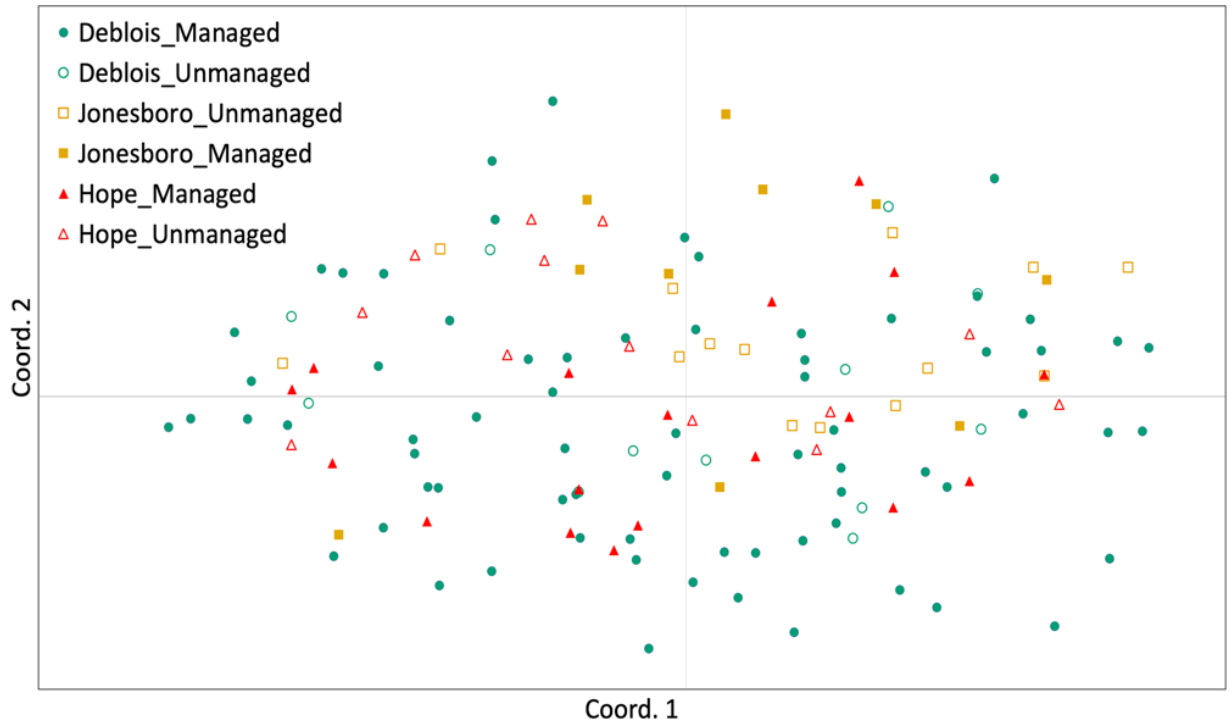


Figure 4.7: Two-dimension plot generated from principal coordinate analysis for six populations (managed and unmanaged fields) from three locations (Deblois, Hope, and Jonesboro) in Maine using 6491 SNP markers.

4.5 Discussion

This is the first study that attempted to apply genome-wide SNP markers to reveal the genetic variation of two wild blueberry species across four locations in Maine. The genetic variation within the wild blueberry population is high, being 94% and 99% in *V. myrtilloides* and *V. angustifolium*, respectively. The genetic differentiation was low among *V. angustifolium* and moderate among *V. myrtilloides* populations. The high within-population genetic variation agrees with the high variation in functional traits within farms and could be partly explained by high within-farm spatial heterogeneity (Barai et al., 2022). Meanwhile, the low or moderate among-population differentiation could be due to seed dispersal by mammals and birds at km distances (Beers et al., 2019). Also, there were no significant genetic differences among unmanaged and managed *V. angustifolium* individuals across the three studied locations in Maine, suggesting that

the current field management practices did not alter the genetic structure of the population, and may not have any negative impact on the genetic diversity of wild blueberry plants.

Genetic Variance and Relationships

An analysis of molecular variance based on 6491 SNPs revealed 99% and 94% variation within populations and 1% and 6% variation among populations of *V. angustifolium* and *V. myrtillodes*, respectively. The high within-population level of genetic variation has been observed in woody perennial shrubs, including wild blueberry (*V. angustifolium)* and Saxaul (*H. ammodendron*) (Sheng et al. 2005; Bell et al. 2012; Beers et al. 2019), which can benefit growers in improving the crop adaptation and resilience to climate change (Hoffmann and Sgrò 2011; Leimu and Fischer 2008). According to Kardos et al. (2021), genetic variation is important for a population to adapt and survive future environmental changes. The wide range of functional traits found in wild blueberry individuals (Barai et al., 2022), increases their chances of being better suited for survival under elevated temperatures currently observed in the fields (Tasnim et al., 2021). In this study, the genetic variance among *V. angustifolium* and *V. myrtillodes* populations was lower than the 8.4% reported among four *V. angustifolium* populations based on EST-PCR-based markers in previous studies (Bell et al. 2009). The difference in findings could be attributed to differences environmental factors of different populations studied or different markers used (Ouborg et al. 1999). The genetic diversity of wild populations can be impacted drastically by changes environmental conditions (Hughes et al. 2008; Gaggiotti et al. 2009). Previous studies have also suggested that long-living, outcrossing woody plant species tend to have low genetic variation among populations (Hamrick et al. 1992).

In this study, PhiPT values (0.008 and 0.064) for *V. angustifolium* and *V. myrtillodes* populations were lower than the values reported in a previous genetic diversity study, where a

PhiPT (0.252) was found in higher population sizes across the Eastern United States, from Maine to North Carolina (Beers et al. 2019). These differences in results could be due to the small population size or the smaller region covered involved in our study. Factors such as small population size and founder effects have been reported to cause low genetic variation (Beers et al. 2019; Frankham 1996; Frankham 2012; Tinnert et al. 2016).

Genetic structure

In our study, there was a random spatial genetic structure in *V. angustifolium* that does not correspond to geographical locations. This result is similar to previous studies, which found a patchy genetic structure of clusters that were randomly distributed in wild blueberry fields (Bell et al. 2009; Bell et al. 2012). According to Drummond (2019), the type of genetic structure exhibited by wild blueberries is attributed to their reproductive biology, as they are primarily obligate outcrossers. Other factors influencing population structure include the seed dispersal mechanism and the pollination system (Drummond 2019). Wild blueberries produce flowers with abundant pollen; however, wild blueberry pollination is by the foraging patterns of some native bees but mostly pollinated by honeybees (Bushmann and Drummond, 2015), and this may have been the reason for the random structure found in this study and previous studies (Bell et al. 2009; Bell et al. 2012).

Genetic variance within wild blueberries results from a complex evolutionary, ecological, and environmental interaction (Ellner 1996; Tinnert et al. 2016). Events including clearing, mowing, and burning of forests are current wild blueberry ecological management practices, which are known to influence the plant population structure (Loveless and Hamrick 1984). Other evolutionary factors, such as latitudinal clines and geographic separation, have increased genetic

distances between species and populations (Howe et al. 1995; Eckert et al. 2008; Friedman et al. 2008).

No genetic Differentiation Between Managed And Unmanaged Fields

Our results showed that there is no clear genetic differentiation between managed and unmanaged wild blueberry fields. This implies that field management may not have an impact on the genetic diversity of plants. However, a study by Beers et al. (2019) has shown that field management is indeed a crucial factor that can affect the genetic diversity of wild blueberries. The reason for the difference in findings could be due to the proximity of unmanaged plants located in the woods, which are often within 100 meters of managed plants. When wild blueberry plants are close enough, they have the opportunity for the exchange of alleles and gene flow through the foraging of bees (Bushmann and Drummond, 2015), which can result in reduced or no genetic differentiation among populations, as observed by Slatkin (1987). Semi-natural systems such as wild blueberry production can benefit from regular irrigation, pruning, burning, application of herbicides, and fertilizer to increase yield and suppress pathogens. However, some field management practices such as burning and pruning were assumed to affect heat-susceptible genotypes, thereby reducing genetic diversity (Smith and Hilton 1971; Hanson et al. 1982). On the other hand, in semi-forest coffee systems, intense management has been found not to cause any negative effect on genetic diversity (Aerts et al. 2013).

4.6 Conclusion

SNP markers, being at a low cost per data point and abundance in genomes, have been successfully used in this study to investigate the population genetic structure of wild blueberries and unravel the extent of variation in commercially managed wild blueberry fields and

unmanaged landscapes. Across different geographical locations, *V. angustifolium* has higher genetic variation within populations than *V. myrtillodes*, asking for further studies to reveal underlying mechanisms. The results from this study corroborate and contribute useful knowledge on the extent of genetic diversity in wild blueberry fields, a semi-natural agricultural system. This information can help to understand the gene flow and seed dispersal for these genetic structures observed among wild blueberry populations. The limited impact of management practices on genetic diversity suggests the naturalness or wildness of this traditional management practice. The high genetic variability in wild blueberry fields can help the wild blueberry production system to resist the impact of increasing climate disturbances and facilitate population adaptation to climate change.

CHAPTER 5

OVERALL CONCLUSIONS AND RECOMMENDATIONS

Climate change affects agricultural production, and the impact is more pronounced on perennial crops. The yield and productivity of perennial crops have been threatened and will continue to be as the human population increases. The impacts of climate warming on the nutritional quality of wild blueberry fruits and bacterial communities of wild blueberry soils were studied, as wild blueberry fields in Maine are experiencing unprecedented, elevated temperatures. I attempted to address four key questions: 1) Will climate warming reduce the nutritional quality of wild blueberries? 2) Can soil amendments mitigate the negative impact of warming on the nutritional quality of wild blueberries? 3) What is the bacterial community composition and diversity of wild blueberry fields under climate warming scenarios compared to ambient conditions? 4) What is the genetic diversity and population structure of wild blueberry crops in managed and unmanaged fields in different regions of Maine?

The results of this study indicate that the concentrations of total soluble solids, fructose, total soluble sugars, and total soluble protein decrease as temperature increases. However, anthocyanin, total flavonoid, and phenolic concentrations remain unaffected. It was observed that sugars such as fructose and glucose are positively correlated with total soluble solids under ambient and passive warming treatments, but not under active warming. Additionally, warming weakens the associations among other chemical compounds. Therefore, future global warming may decrease the sweetness of wild blueberries, which could impact their taste and nutritional content. A consumer acceptance study of wild blueberries under different scenarios is recommended to determine the effect of climate change on consumer preference and human nutrition. Furthermore, readily available soil management practices such as mulching and

biochar should be tested on wild blueberry fields under warming conditions to mitigate any negative impact.

The results of this study also indicate the detrimental impact of warming on the nutritional qualities of wild blueberries could not be fully mitigated by mulching and biochar-compost applications. Only potassium and magnesium concentrations were positively affected. To fully understand how elevated temperatures impact wild blueberry nutrition, we need to understand the underlying physiological and biochemical responses to warming better. Further research is required to determine whether increasing the application rate of biochar-compost mix and mulching or other agricultural approaches can mitigate the adverse effects of warming. As such, wild blueberry researchers and growers need to continue assessing the risks of climate change, developing preparedness plans, and engaging key stakeholders on a more climate-smart approach to mitigating its effects.

Through profiling the response of soil bacterial composition and diversity to climate warming using 16S rRNA gene sequencing, it has been determined that climate warming affects the diversity and composition of bacteria in wild blueberry soils during the early growing seasons. The shift in bacterial communities due to warming is more pronounced in June, and the increased bacteria evenness and diversity may be linked to advanced plant phenology. Genus *Acidothermus*, a cellulolytic bacterium, is more abundant in warm soils and can help recycle plant biomass more quickly. The study also found that seasonal changes in temperature and plant activity impact bacterial community structure more than the warming treatments. Some members of *Variovorax*, *Acidothermus*, *Bryobacter*, *Candidatus Xiphinematobacter*, and *Aquisphaera* are essential predictors of bacterial abundance in wild blueberry farms under elevated temperatures. The dominant genus *Acidothermus* interacts positively with other

microbes to promote soil health and increase wild blueberry above-ground biomass. This study provides insight into the effect of elevated temperatures on the composition and diversity of bacteria in wild blueberry soils.

Genome-wide SNP markers were employed to successfully examine the genetic variation of two wild blueberry species (*Vaccinium angustifolium* and *Vaccinium myrtilloides*) across four locations (Hope, Jonesboro, Deblois, and Fort Kent) in Maine, spanning 350 km. The study found that the genetic variation within the wild blueberry population is high, at 94% and 99% in *V. myrtilloides* and *V. angustifolium*, respectively. This high within-population genetic variation agrees with the high variation in functional traits within farms, which could be explained by high within-farm spatial heterogeneity. The genetic differentiation was low among *V. angustifolium* and moderate among *V. myrtilloides* populations, which could be attributed to seed dispersal by mammals and migratory birds over distances of kilometers. This information is useful in understanding the gene flow and seed dispersal of wild blueberry populations.

Additionally, the study found no significant genetic differences between managed and unmanaged *V. angustifolium* individuals across three locations in Maine, indicating that current field management practices did not alter the genetic structure of the population and are not likely to have a negative impact on the genetic diversity of wild blueberry plants. This study suggests that traditional management practices maintain the naturalness or wildness of the system. The high genetic variability in wild blueberry fields can help these plants to resist the impact of increasing climate disturbances and facilitate population adaptation to climate change.

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