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Effects of Nitrogen Fertilization on the Phenolic Composition and Antioxidant Properties of Basil (*Ocimum basilicum* L.)

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Abstract

Many herbs and spices have been shown to contain high levels of polyphenolic compounds with potent antioxidant properties. In the present study, we explore how nutrient availability, specifically nitrogen fertilization, affects the production of polyphenolic compounds in three cultivars (Dark Opal, Genovese, and Sweet Thai) of one of the most common culinary herbs, basil (*Ocimum basilicum L.)*. Nitrogen fertilization was found to have a significant effect on total phenolic levels in Dark Opal ($p < 0.001$) and Genovese ($p < 0.001$) basil with statistically higher phenolic contents observed when nutrient availability was limited at the lowest (0.1 mM) applied nitrogen treatment. Similarly, basil treated at the lowest nitrogen fertilization level generally contained significantly higher rosmarinic ($p = 0.001$) and caffeic ($p = 0.001$) acid concentrations than basil treated at other nitrogen levels. Nitrogen fertilization also affected antioxidant activity ($p = 0.002$) with basil treated at the highest applied nitrogen level, 5.0 mM, exhibiting lower antioxidant activity than all other nitrogen treatments. The anthocyanin content of Dark Opal basil was not affected by applied nitrogen level, but anthocyanin concentrations were significantly impacted by growing season ($p = 0.001$). Basil cultivar was also determined to have a statistically significant effect on total phenolic levels, rosmarinic and caffeic acid concentrations, and antioxidant activities.

Introduction

Epidemiological evidence increasingly suggests that consumption of a diet rich in plant foods has a protective effect against cardiovascular disease and certain forms of cancer (*1-3*). Although plants contain a variety of components which may lead to their overall health benefits including proteins, amino acids, vitamins, and fiber, recent research has focused on the role of secondary plant metabolites, particularly polyphenolic compounds and flavonoids, in disease prevention (*3*). Plant polyphenols can vary widely in their structure and general classification but all share the common feature of containing at least one aromatic ring and one or more hydroxyl groups. Polyphenolic compounds in plants are naturally-occurring antioxidants and their radical scavenging capabilities are thought to play an important function in preventing many chronic illnesses (*4-6*). Plant polyphenols have been shown to inhibit angiogenesis,

tumorigenesis, and metastasis (*7-9*) and many are known to have antibacterial, antifungal and anti-inflammatory capabilities (*10*).

Although the nutritional benefits derived from eating polyphenol-rich plant foods are well known, foods and beverages containing the highest polyphenolic levels (such as soy products or green tea) are often lacking or absent in many diets, particularly in Western countries (*11, 12*). Therefore, there has been growing interest in developing simple methodologies to increase polyphenol concentrations in more commonly consumed plant foods to further enhance their overall nutritional value (*13-15*). Polyphenolic compounds are produced by plants throughout their development for a variety of reasons: defense against microorganisms, insects, or herbivores (*16, 17*); nutrient availability (*17*); exposure to ultraviolet radiation (*18*); and because of allelopathic interactions (*19*). However, because plant responses to such stimuli are highly varied and not well understood, utilizing such techniques to induce plants to produce secondary metabolites (therefore potentially increasing their nutritional value) is not common.

In particular, the availability of key macronutrients during plant growth has significant potential to affect polyphenolic accumulation (*15*). Though nitrogen, phosphorus, potassium, and calcium fertilization levels have been shown to affect the production of secondary metabolites in some plants (*20-24*), mineral nutrition has little or no effect on polyphenolic production in others (*25-27*).

In the present study, we explore how nutrient availability, specifically nitrogen fertilization, affects the production of polyphenolic compounds in *Ocimum basilicum* L. (basil). Although nitrogen fertilization has been previously shown to directly correlate with the growth, yield, and essential oil content of basil (*28, 29*), the effect of nitrogen availability on the polyphenolic composition and antioxidant properties of basil has not yet been determined.

We have chosen basil for our study because it is one of the most popular culinary herbs worldwide, has a variety of cultivars available, and is often commercially produced in greenhouses, allowing for controlled manipulation of growing conditions (*30*). Moreover, basil produces a range of polyphenolic compounds including rosmarinic acid, a characteristic it shares with herbs in the genus *Lamiaceae*. Rosmarinic acid is a cinnamic acid derivative with potent antioxidant activity (*31*) and known antiviral, antibacterial, and anti-inflammatory properties (*32*). In addition, several purple basil cultivars also contain anthocyanins (*33*) which are powerful antioxidants (*34*), and the polyphenolic pigments responsible for the red and blue colors found in many plants (*35*).

Materials and Methods

Chemicals. All standards such as phenolic acids (e.g., rosmarinic acid, gallic acid, and caffeic acid) and anthocyanins (e.g., kuromanin chloride) were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO). General reagents (such as 2,2'-diphenyl-1-picrylhydrazyl (DPPH), formic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), etc.) were purchased from either Fisher Scientific (Springfield, NJ) or Sigma-Aldrich. All solvents were HPLC/UV grade and were purchased from Pharmco Products Inc. (Brookfield, CT). Phenol reagent was obtained from VWR (Suwanee, GA). Reagent-grade salts for the preparation of nutrient watering solutions were purchased from Sigma-Aldrich and used without further purification.

Plant Materials and Growth Conditions. Basil plants were grown in a greenhouse at Southwestern University (Georgetown, TX) under natural temperature and light conditions. Three different basil cultivars, Dark Opal, Genovese, and Sweet Thai, were grown from seed (Johnny's Seeds, Winslow, ME) during two planting seasons: summer (Dark Opal and Sweet

Thai sown on June 14, 2006) and fall (Dark Opal and Genovese sown on October 5, 2006). All plants were germinated and grown in course sand in 1 gallon black plastic pots. Modified Hoagland solutions were prepared with deionized water at 4 different nitrogen treatment levels with all other macro- and micronutrient concentrations held constant. Watering solutions contained the following nutrient levels (mM): N (as ammonium nitrate, 0.1, 0.5, 1.0 or 5.0); S (3.0); K (2.0); Mg (2.0); P (2.0); Ca (1.0); Cl (0.05); Fe (0.04 as Fe-EDTA); B (0.033); Mn (0.002); Zn (0.002); Cu (0.0005); Mo (0.0005). During each growing season, basil plants were arranged in a complete randomized block design based on the four nitrogen fertilization levels, two basil cultivars, and 5 replicates for each treatment (number of pots = 40 per season; 80 total for this study). Basil seeds and plants were watered daily with approximately 125 mL of the appropriate modified Hoagland nutrient solution. Temperatures within the greenhouse ranged from 18 – 31 ^{o}C during the summer growing season and 14 – 30 ^{o}C during the fall. All basil plants were harvested together 30 days after germination, frozen in liquid nitrogen, and immediately stored at -80 ^{o}C until use.

For two of the basil cultivars, the extreme nitrogen treatment levels resulted in death of all replicate plants prior to harvest. Therefore, samples were not collected for Sweet Thai treated with 0.1 mM nitrogen and Genovese treated with 5.0 mM nitrogen. Dark Opal basil plants which did not exhibit purple leaves at the time of harvest (~ 20% of the variety is variegated or green), were excluded from further analysis.

Sample Preparation. For each plant, basil leaves of uniform size were ground in liquid nitrogen using a mortar and pestle and 0.10 – 0.15 g samples were dried for 2 hrs using vacuum centrifugation. The dried basil (weighing 0.01 – 0.015 g) was then mixed with 1.0 mL of 80% aqueous methanol and shaken for 15 hrs at room temperature to extract phenolic compounds.

The mixture was centrifuged at 12,000 rpm for 20 min and the extract was stored at -80 ^{o}C until analysis. To extract anthocyanins, 1.0 mL of acidified methanol (15% HCl v/v) was added to the dried Dark Opal basil (weighing 0.01 – 0.015 g) in a black microcentrifuge tube to minimize photodegradation of the sample (*36*). The mixture was shaken for 30 min at room temperature, centrifuged at 13,200 rpm for 30 min, and the extract was stored at -80 ^{o}C until analysis (*37*). A modification (*38*) of the spectrophotometric pH differential method (*39*) was used to confirm that the extraction process did not lead to anthocyanin degradation in the basil samples.

Determination of Total Phenolic Compounds. A modified version of the Folin-Ciocalteu colorimetric assay (*40*) was used to determine the total phenolic content of all basil samples. Briefly, methanolic basil extract (50.0 μL), deionized water (450.0 μL), Folin-Ciocalteu phenol reagent (250.0 μL) and 20% sodium carbonate (1.25 mL) were added to an amber vial, mixed, and allowed to incubate at room temperature for 20 min. Absorbance of the samples was then measured at 735 nm against a distilled water/sodium carbonate blank. The total phenolic content in each sample (expressed as gallic acid equivalents, GAE, in mg/g dried basil) was quantified by comparing the absorbance of the basil extract against a standard curve prepared with gallic acid.

Quantification of Total Anthocyanins. Total anthocyanins were determined spectrophotometrically for Dark Opal basil samples using a method previously developed by Abdel-Aal and Huel (*37*). The anthocyanin-containing basil extract (125 μL) was diluted to 1.0 mL with acidified methanol (15% HCl v/v) and its absorbance was measured at 535 nm against a reagent blank. Total anthocyanin concentration (expressed as mg anthocyanin equivalents, AE, per g dried basil) in the extract was then determined by comparing the sample absorbance to a standard curve prepared with kuromanin chloride (cyanidin 3-glucoside) in acidified methanol.

Determination of Antioxidant Activities. The antioxidant activity of each basil sample was determined using a modified version of the DPPH free radical scavenging assay as described by Kim *et al.* (*41*). Methanolic basil extract (25.0 μL for Dark Opal, 5.0 μL for Sweet Thai, 20.0 μL for Genovese) was diluted to 100 μL with 80% aqueous methanol and added to 0.4 mL of 0.1M Tris-HCl buffer and 0.5 mL of 0.3 mM DPPH in methanol. The solution was mixed thoroughly and incubated in the dark for 20 min at room temperature. The absorbance of the sample mixture (A_{sample}) was monitored at 517 nm versus a methanol/Tris-HCl blank. The absorbance of a control sample ($A_{control}$) containing only 80% methanol, Tris-HCl, and DPPH was also analyzed. The % DPPH free radical scavenging activity was calculated according to the following equation:

$$\%\ \text{DPPH free radical scavenging} = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100$$

The antioxidant activity was then determined by comparing the % DPPH free radical scavenging of each basil sample to a calibration curve prepared with trolox, a well-known antioxidant standard. Antioxidant activities were expressed as the trolox equivalent antioxidant capacity (TEAC, mmol of trolox equivalents/g dried basil) to allow direct comparison of the free radical scavenging capabilities between all basil samples.

HPLC Analysis of Individual Phenolic Compounds. Rosmarinic acid and caffeic acid were quantified in all basil samples using a dual-pump Waters HPLC system (Milford, MA) equipped with a 20 μL injection loop and a Waters Symmetry C-18 column (5 μm, 4.6 × 150 mm). The method was based on separation conditions initially developed by Shan *et al.* (*42*) using 2.5% aqueous formic acid (eluent A) and 100% acetonitrile (eluent B) but incorporated a significantly reduced analysis time (40 min). The following linear gradient was used at a mobile

phase flow rate of 1 mL/min with detection at 330 nm: 85% A, 0 min; 75% A, 15 min; 70% A, 20 min; 45% A, 24 min; 10% A, 28 min; 0% A, 30 min; 85% A, 35 – 40 min. Each basil sample extract was diluted with 80% aqueous methanol and filtered using a Whatman 0.45 μm nylon filter prior to analysis. Rosmarinic acid and caffeic acid were identified in basil samples based on their chromatographic retention times and quantified by comparing integrated peak areas to calibration curves prepared with analytical standards.

Identification of Phenolic Compounds with Radical Scavenging Activity. Methanolic basil extract (75 μL) was diluted to 100 μL with 80% aqueous methanol and added to 0.4 mL of 0.1M Tris-HCl buffer and 0.5 mL of 0.3 mM DPPH in methanol. The solution was thoroughly mixed then incubated at room temperature for 20 min. After filtration using a Whatman 0.45 μm nylon filter, the sample was directly injected into the HPLC using the analytical method described above. The resulting chromatograms were then compared to those obtained for the pure basil extract. The fraction of the remaining peak area to the initial area was calculated for rosmarinic acid and caffeic acid (*43*).

Data and Statistical Analyses. One-way analysis of variance (ANOVA) was used to determine whether nitrogen treatment, cultivar, and season had a statistically significant impact on total phenolic levels, anthocyanin concentrations, antioxidant activities, and rosmarinic and caffeic acid levels for each of the basil cultivars. Statistical significance was determined at the $p < 0.05$ level using Tukey's post hoc test. Two-way ANOVA was used to elucidate whether combined effects between nitrogen fertilization level and basil cultivar existed for total phenolic contents and antioxidant activities. All statistical analyses were completed using SPSS, version 13.0 (SPSS Inc., Chicago, IL).

Results and Discussion

Analysis of Total Phenolics and Total Anthocyanins. The average total phenolic contents of Sweet Thai, Dark Opal, and Genovese basil grown with varying nitrogen fertilization levels are presented in Figure 1. Total phenolic concentrations ranged from 7 mg GAE/g of dry weight (DW) for Sweet Thai treated with 5.0 mM nitrogen to 31 mg GAE/g DW for summer-grown Dark Opal with 0.1 mM applied nitrogen. Average total phenolic contents were found to be 20.95 (± 11.90) mg GAE/g DW for Dark Opal, 16.60 (± 4.17) mg GAE/g DW for Genovese, and 10.66 (± 5.65) mg GAE/g DW for Sweet Thai. The total phenolic levels determined for basil cultivars in this study are therefore similar in magnitude to those found in previous analyses of sweet basil: 7 (*44*), 26 (*45*), and 36 (*42*) mg GAE/g DW.

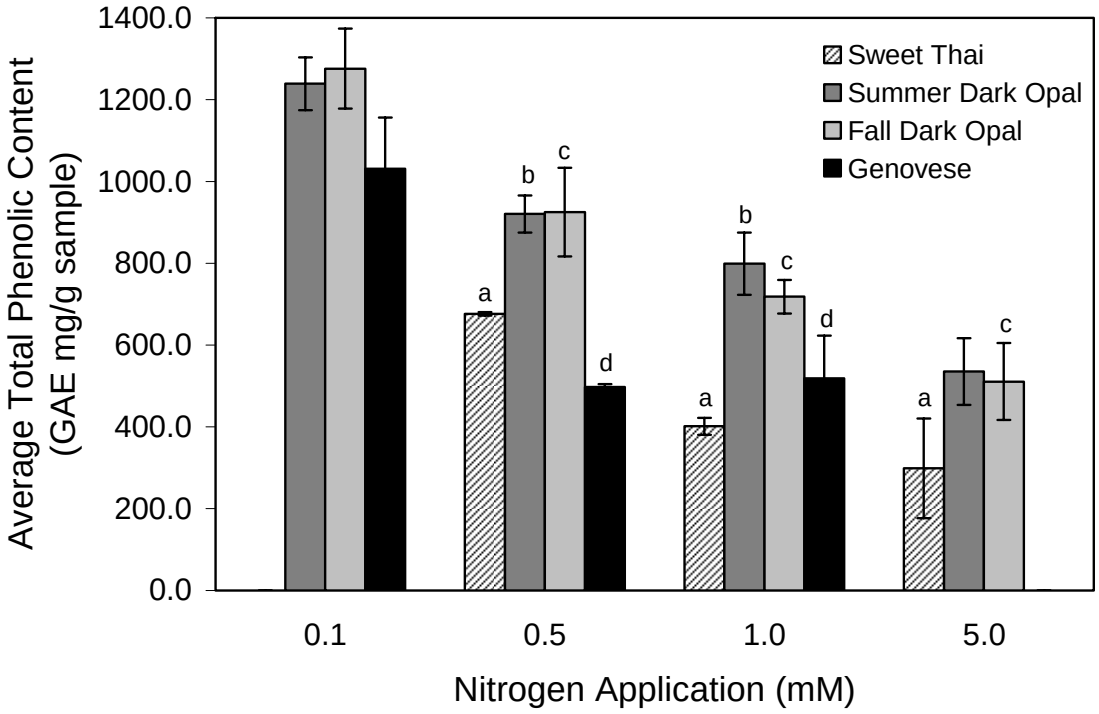


Figure 1. Average total phenolic contents for Sweet Thai, Dark Opal (planted in summer and fall), and Genovese basil as a function of applied nitrogen level. All concentrations are expressed as gallic acid equivalents, GAE, in mg/g DW. Error bars represent the standard deviations calculated from the analysis of replicate plant samples. Total phenolic contents with the same letter for a given basil cultivar are not statistically different ($p < 0.05$).

Nitrogen fertilization was found to have a statistically significant effect on total phenolic levels in Dark Opal ($p < 0.001$) and Genovese ($p < 0.001$) basil. For the summer planting of Dark Opal, a significant difference in total phenolic concentrations was observed for all nitrogen fertilization treatments except 0.5 and 1.0 mM ($p = 0.214$), with 0.1 mM applied nitrogen leading to the highest total phenolic levels while 5.0 mM applied nitrogen produced the lowest. For Genovese basil and the fall planting of Dark Opal, 0.1 mM nitrogen fertilization caused the production of statistically higher total phenolic levels than all other nitrogen treatments. In contrast, the total phenolic content of Sweet Thai basil was not significantly influenced by applied nitrogen ($p = 0.964$), but this result is likely influenced by the inability to grow Sweet Thai basil at the lowest nitrogen treatment level (0.1 mM) in this study.

Our results may be explained using the growth-differentiation balance (GDB) framework which is based on the principle that a "physiological trade-off" exists between plant growth and secondary metabolite production (*17*). Nitrogen is an essential soil-derived macronutrient that is needed in relatively large amounts by plants for adequate growth as well as amino acid, enzyme, and protein formation (*46*). When environmental conditions are good and nitrogen levels are adequate, the GDB theory states that plant growth will be favored, with production of photosynthetic proteins receiving resource priority. However, when environmental conditions are poor and the availability of an essential nutrient such as nitrogen is limited, the GDB framework proposes that growth allocation for a plant will decrease while the production of secondary metabolites that may aid in storage and defense subsequently increase (*17*).

Within the GDB framework, the carbon/nutrient balance (CNB) hypothesis (*47*) more specifically addresses the effects of fertilization on plant resource allocation. The CNB theory states that under limited nutrient conditions, plants increase their production of carbon-based

compounds, particularly secondary metabolites. Based on the CNB hypothesis, one would therefore expect low nitrogen fertilization levels to lead to increased concentrations of carbonaceous metabolites such as polyphenolic compounds. Although some previous studies have shown that conditions may exist in which nutrient availability does not influence secondary metabolite production (*25-27*), our results for Genovese and Dark Opal basil directly support the CNB hypothesis: significantly higher phenolic levels are observed when nutrient availability is limited at the lowest (0.1 mM) applied nitrogen treatment. Furthermore, for Dark Opal basil grown in the summer, significantly lower phenolic levels were determined for the nutrient-rich conditions at the highest applied nitrogen treatment (5.0 mM).

Cultivar was also found to have a statistically significant impact on total phenolic levels. Anthocyanin-containing Dark Opal basil had higher phenolic content than the green Genovese ($p = 0.006$) and Sweet Thai ($p < 0.001$) varieties, with Sweet Thai basil having the lowest total phenolic content overall. Cultivar is known to strongly influence the expression of polyphenolic compounds in a variety of plants (*15*) and basil genotypes have been previously reported to have large variations in their chemical composition (*30*), although most studies have focused on essential oil composition (*48*) rather than foliar phenolic concentrations. Although nitrogen fertilization and cultivar were both found to influence total phenolic levels in basil, no interaction was found between these two variables ($p = 0.620$).

Table 1 presents the average total anthocyanin concentrations for Dark Opal basil grown in summer and fall with varying nitrogen fertilization treatments. Anthocyanin concentrations in Dark Opal basil ranged from 7 mg AE/g DW (grown in summer with 0.5 and 5.0 mM applied nitrogen) to 14 mg AE/g DW (grown in fall with 0.1 and 0.5 mM applied nitrogen). Although a

previous study showed much lower anthocyanins levels of 0.16 to 0.18 mg AE/g in Dark Opal basil (*33*), these values were determined in fresh leaves rather than dried.

Table 1. Average anthocyanin concentrations and standard deviations[a] for Dark Opal basil planted in summer and fall as a function of applied nitrogen level. All concentrations are expressed as anthocyanin equivalents, AE, in mg/g DW.

	Nitrogen Application (mM)			
	0.1 mM	0.5 mM	1.0 mM	5.0 mM
Summer Dark Opal (mg AE/g)[b]	8.33 ± 3.45 a	7.29 ± 3.56 a	9.51 ± 0.84 a	7.17 ± 2.32 a
Fall Dark Opal (mg AE/g)[b]	14.72 ± 1.96 b	14.46 ± 1.18 b	11.16 ± 1.19 b	12.55 ± 4.17 b

[a] Standard deviations are calculated from the analysis of replicate plant samples.
[b] Concentrations with the same letter in each row are not statistically different ($p < 0.05$).

Nitrogen fertilization was determined to have no significant effect on the anthocyanin content of Dark Opal basil grown in either summer or fall. However, anthocyanin levels were significantly impacted by season ($p = 0.001$), with Dark Opal basil grown in the fall having statistically higher anthocyanin content. Anthocyanins have many diverse roles in plants, but their production is known to be associated with protection against environmental stresses (*49, 50*). In particular, anthocyanins are induced by colder temperatures (*50*), so their increased concentration in fall-grown Dark Opal basil most likely results from the lower daily and nightly temperatures that occured during the autumn growing season.

Quantification of Individual Basil Phenolics. A typical chromatogram of methanolic basil extract is presented in Figure 2A. The largest chromatographic peak at a retention time of 11.3 min is rosmarinic acid, which is known to be the free phenolic acid present in highest concentration in *Ocimum basilicum* (*51*). Caffeic acid, which is also commonly present in moderately high concentrations in basil (*51*), is identified at a retention time of 3.6 min.

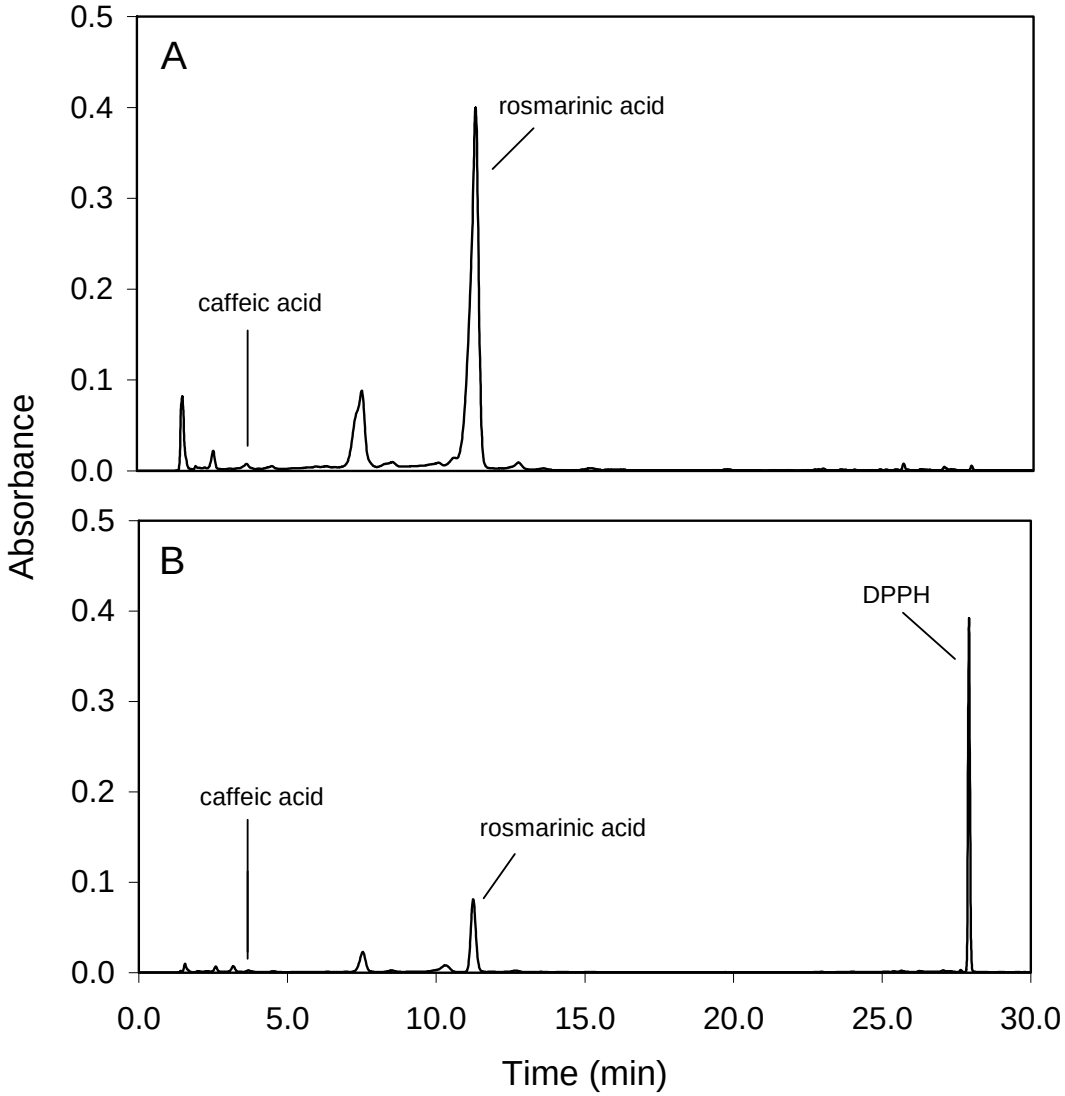


Figure 2. Typical HPLC chromatogram of methanolic basil extract before (A) and after the addition of DPPH free radical scavenger (B).

Table 2 presents the average rosmarinic acid concentrations of Sweet Thai, Dark Opal and Genovese basil grown with varying nitrogen fertilization levels. Rosmarinic acid levels ranged from 5 mg/g DW for Sweet Thai treated with 5.0 mM applied nitrogen to 48 mg/g DW for summer-grown Dark Opal treated with 0.1 mM nitrogen. The rosmarinic acid levels obtained for basil cultivars in this study are similar to concentrations determined previously for sweet basil: 11 (*42*), 12 (*51*), and from 10 to 100 mg/g DW (*52*). Herbs in the family *Lamiaceae*, such

as basil, rosemary, sage, and thyme, provide the only dietary source of rosmarinic acid (*53*), with concentrations typically ranging from 2 to 27 mg/g DW (*54*).

Table 2. Average rosmarinic acid concentrations and standard deviations[a] for basil as a function of applied nitrogen level. All rosmarinic acid concentrations are expressed in mg/g DW.

	Nitrogen Application (mM)			
Cultivar	0.1 mM	0.5 mM	1.0 mM	5.0 mM
Sweet Thai[b] (mg/g)	—	17.59 ± 2.12 a	12.06 ± 2.69 ab	5.41 ± 0.86 b
Summer Dark Opal[b] (mg/g)	47.89 ± 17.13 a	25.77 ± 0.14 a	20.57 ± 0.86 a	12.04 ± 2.87 a
Fall Dark Opal[b] (mg/g)	45.21 ± 2.95 a	18.16 ± 1.91 b	16.25 ± 3.33 b	11.45 ± 1.77 b
Genovese[b] (mg/g)	23.55 ± 3.84 a	9.82 ± 0.64 b	12.00 ± 1.43 b	—

[a] Standard deviations are calculated from the analysis of replicate plant samples.
[b] Concentrations with the same letter in each row are not statistically different ($p < 0.05$).

Average rosmarinic acid concentrations were found to be 25.00 (± 7.73) mg/g DW for Dark Opal, 15.12 (± 2.44) mg/g DW for Genovese, and 13.51 (± 1.77) mg/g DW for Sweet Thai basil. Cultivar had a significant effect on rosmarinic acid levels in basil, with both Sweet Thai and Genovese varieties having statistically lower rosmarinic acid concentrations than summer-grown Dark Opal basil ($p = 0.005$). In addition, Dark Opal basil grown in summer and fall was compared to determine the effect of season on rosmarinic acid levels. Although rosmarinic acid concentrations tended to be higher in summer-grown Dark Opal basil, our data indicated that growing season does not have a statistically significant effect on the amount of rosmarinic acid found in basil ($p = 0.472$).

Rosmarinic acid concentrations in basil were significantly affected by nitrogen fertilization and, in general, basil treated with 0.1 mM applied nitrogen contained higher rosmarinic acid content than basil treated at other nitrogen fertilization levels ($p = 0.001$).

Comparison of the effect of applied nitrogen on rosmarinic acid levels among individual cultivars showed that a greater amount of rosmarinic acid was found at the lowest nitrogen fertilization level for fall-grown Dark Opal ($p < 0.001$), Genovese ($p = 0.014$), and Sweet Thai ($p = 0.014$) basil. Dark Opal grown in the summer, on the other hand, did not contain rosmarinic acid concentrations that were statistically higher for the 0.1 mM applied nitrogen level ($p = 0.179$). Although the concentration of rosmarinic acid in summer-grown Dark Opal was found to be the greatest for 0.1 mM applied nitrogen, high variability excluded the result from being statistically significant.

Average caffeic acid concentrations in Dark Opal, Genovese, and Sweet Thai basil grown with varying nitrogen fertilization levels are presented in Table 3. Caffeic acid concentrations ranged from 0.113 mg/g DW for Sweet Thai basil treated with 1.0 mM applied nitrogen to 0.556 mg/g DW for summer-grown Dark Opal basil treated at the 0.1 mM nitrogen fertilization level. Previous studies of caffeic acid concentrations in sweet basil have obtained values of less than 0.004 mg/g DW (*52*) to greater than 2.5 mg/g DW (*51*). In a study of 23 accessions found in different regions of Iran, Javanmardi *et al.* observed large variability in basil caffeic acid concentrations, and suggested that it may be due to changes in biosynthetic pathways caused by environmental fluctuations (*52*), making comparison of our basil caffeic acid levels to previous studies difficult.

Table 3. Average caffeic acid concentrations and standard deviations[a] for basil as a function of applied nitrogen level. All caffeic acid concentrations are expressed in mg/g DW.

	Nitrogen Application (mM)			
Cultivar	0.1 mM	0.5 mM	1.0 mM	5.0 mM
Sweet Thai[b] (mg/g)	—	0.228 ± 0.037 a	0.113 ± 0.026 a	0.150 ± 0.056 a
Summer Dark Opal[b] (mg/g)	0.556 ± 0.143 a	0.141 ± 0.000 a	0.301 ± 0.077 a	0.372 ± 0.047 a
Fall Dark Opal[b] (mg/g)	0.464 ± 0.047 a	0.186 ± 0.040 b	0.239 ± 0.041 b	0.152 ± 0.016 b
Genovese[b] (mg/g)	0.195 ± 0.012 a	0.248 ± 0.014 a	0.328 ± 0.059 a	—

[a] Standard deviations are calculated from the analysis of replicate plant samples.
[b] Concentrations with the same letter in each row are not statistically different ($p < 0.05$).

The average concentrations of caffeic acid were found to be 0.301 (± 0.185) mg/g DW in Dark Opal, 0.257 (± 0.06) mg/g DW in Genovese, and 0.164 (± 0.072) mg/g DW in Sweet Thai basil. These values are similar in magnitude to the caffeic acid levels quantified by Shan *et al*. in sweet basil, 0.204 mg/g DW (*42*). As we observed for rosmarinic acid levels and total phenolic contents, cultivar had a significant effect on the amount of caffeic acid in basil ($p = 0.030$), with Sweet Thai having the least caffeic acid while Dark Opal had the most. Caffeic acid levels tended to be generally higher for basil grown in the summer than in the fall, however, the difference was not found to be statistically significant ($p = 0.117$). Although the effect of growing season on phenolic compounds such as caffeic acid has not been extensively studied in basil, recent work has shown that season has a statistically significant impact on caffeic acid levels in other plants such as napiergrass (*55*).

Nitrogen fertilization had a statistically significant effect on caffeic acid levels, with 0.1 mM applied nitrogen generally resulting in higher concentrations of caffeic acid than basil treated at other nitrogen levels ($p = 0.001$). Although the highest caffeic acid concentrations

were observed for the lowest nitrogen fertilization levels for fall (0.464 ± 0.143 mg/g DW) and summer-grown (0.556 ± 0.143 mg/g DW) Dark Opal as well as Sweet Thai basil (0.228 ± 0.037 mg/g DW), Genovese basil contained the highest caffeic acid content (0.328 ± 0.059 mg/g DW) in basil treated at the highest applied nitrogen level, 1.0 mM.

Based on the CNB hypothesis (*17, 47*), limited nutrient availability increases the production of carbon-based secondary plant metabolites, particularly those that accumulate in plant tissue at high concentrations since they are often the stable end products of biochemical pathways directly associated with resource allocation (*17*). Therefore, the fact that the highest rosmarinic and caffeic acid concentrations in basil are generally observed at the lowest nitrogen fertilization levels is directly supported by the CNB theory. Furthermore, because the highest total phenolic contents were found when nutrient availability was limited at the lowest applied nitrogen treatment, it is expected that the highest concentrations of primary individual phenolics such as rosmarinic and caffeic acids would also be observed under those conditions.

Determination of Antioxidant Activities. Average antioxidant activities for Sweet Thai, Dark Opal, and Genovese basil as a function of nitrogen fertilization were determined using the DPPH radical scavenging assay and are presented in Table 4. Antioxidant activities ranged from 5 mmol TEAC/100 g DW for Sweet Thai treated with 5.0 mM applied nitrogen to 40 mmol TEAC/100 g DW for Dark Opal grown in the summer with 0.5 mM nitrogen fertilization. Literature values for the antioxidant activity of sweet basil determined by the DPPH assay range from 23 (*45*) to 30 (*42*) mmol TEAC/100 g DW and are in good agreement with our values in the current study.

Table 4. Average antioxidant activities and standard deviations[a] for Sweet Thai, Dark Opal (planted in summer and fall), and Genovese basil as a function of applied nitrogen level. All concentrations are expressed as the trolox equivalent antioxidant capacity, TEAC, in mmol/100 g DW.

	Nitrogen Application (mM)			
Cultivar[b]	0.1 mM	0.5 mM	1.0 mM	5.0 mM*
Sweet Thai* (mmol TEAC/100 g)	—	27.6 ± 7.7	11.56 ± 4.90	5.23 ± 0.69
Summer Dark Opal (mmol TEAC/100 g)	34.51 ± 3.18	40.29 ± 16.16	36.22 ± 8.01	20.17 ± 9.74
Fall Dark Opal (mmol TEAC/100 g)	24.80 ± 4.41	27.41 ± 2.89	27.87 ± 1.52	24.59 ± 6.87
Genovese (mmol TEAC/100 g)	26.56 ± 2.10	29.17 ± 1.36	28.48 ± 0.66	—

[a] Standard deviations are calculated from the analysis of replicate plant samples.
[b] * denotes a significant difference at the $p < 0.05$ level.

Cultivar was found to have a significant effect on antioxidant activity ($p < 0.001$) with Sweet Thai having lower antioxidant activity than Dark Opal and Genovese basil. Antioxidant activity correlates directly with total phenolic content (*56*), so it is expected that the basil cultivar exhibiting the lowest total phenolic levels, Sweet Thai, would also have the lowest overall antioxidant activity.

Nitrogen fertilization also affected antioxidant activity ($p = 0.002$) with basil treated at the highest applied nitrogen level, 5.0 mM, exhibiting significantly lower antioxidant activity than all other nitrogen treatments. The low antioxidant activities determined for basil treated with 5.0 mM applied nitrogen likely relate directly to the low total phenolic contents found for the same samples (see Figure 1). Although the 0.1 mM nitrogen-treated basil exhibited the highest total phenolic levels overall, these basil samples did not have statistically higher antioxidant activities, but this may be due to the variability in the data for antioxidant capacities determined for replicate plant samples. Although both nitrogen fertilization and cultivar were

found to impact basil antioxidant activity, no interaction was found between the two factors (p = 0.290).

To determine the radical scavenging activity of the individual basil phenolics, methanolic extract was analyzed by HPLC (Figure 2B) after performing a free radical scavenging assay (*43*). Rosmarinic and caffeic acid levels were quantified after the addition of DPPH and expressed as the percentage of phenolic acid remaining after radical scavenging (Table 5). Small amounts of the phenolic acids remained after completing the assay, indicating a high rate of DPPH free-radical scavenging activity by the basil extract.

Table 5. Average rosmarinic and caffeic acid concentrations and standard deviations[a] (expressed in mg/g DW) before adding DPPH, and the percentage of phenolics remaining following the DPPH free-radical scavenging assay. Data is shown for basil treated with 1.0 mM applied nitrogen.

	Initial Phenolic Concentration		Percent Remaining	
Cultivar	RA (mg/g DW)	CA (mg/g DW)	% RA[b]	% CA[b]
Sweet Thai	13.51 ± 1.77	0.16 ± 0.07	7.73 ± 0.19	14.66 ± 0.95
Summer Dark Opal	26.57 ± 17.4	0.34 ± 0.17	7.46 ± 0.53	30.07 ± 9.85
Fall Dark Opal	22.77 ± 5.15	0.26 ± 0.08	4.74 ± 1.05	12.37 ± 0.09
Genovese	15.12 ± 2.44	0.26 ± 0.06	6.80 ± 0.95	10.88 ± 0.59

[a] Standard deviations are calculated from the analysis of replicate plant samples.
[b] The percentages of rosmarinic and caffeic acid were calculated by dividing the initial phenolic concentrations by the amounts of phenolic acid remaining after the DPPH free-radical scavenging assay.

Rosmarinic and caffeic acid concentrations decreased significantly after DPPH free-radical scavenging for all cultivars. However, the decrease in rosmarinic acid levels (4 – 7% remaining after radical scavenging) was greater than the decrease in caffeic acid concentrations (10 – 30% remaining after radical scavenging). This result suggests that the DPPH free-radical

scavenging activity of rosmarinic acid in our basil extracts is greater than that of caffeic acid. Our results are supported by a previous study by Chen and Ho (*31*) which found that rosmarinic acid had a greater DPPH radical scavenging capacity than caffeic acid. In addition, the reaction of rosmarinic acid with DPPH has been shown to exhibit intermediate kinetic behavior while the reaction of caffeic acid with DPPH is kinetically slow (*57*).

Conclusions

Our results indicate that manipulation of nitrogen fertilization levels may be an effective method to increase the expression of polyphenolic compounds in basil. Higher total phenolic contents, rosmarinic acid levels, and caffeic acid concentrations were observed in basil when nutrient availability was limited at the lowest applied nitrogen treatment. Moreover, at the highest nitrogen treatment level, basil exhibited significantly lower antioxidant activity than under limited nutrient growing conditions. In addition to nitrogen fertilization, cultivar selection was also found to have a significant influence on polyphenolic content and antioxidant activity in basil.

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