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Effect of nitrogen, location, and harvesting stage on peppermint (Mentha X piperita L.) productivity, oil content, and composition

Vasile Cerven

EFFECT OF NITROGEN, LOCATION, AND HARVESTING STAGE ON PEPPERMINT (*Mentha X piperita* L.) PRODUCTIVITY, OIL CONTENT, AND COMPOSITION

By

Vasile Cerven

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Approved:

_____	_____
Valtcho D. Jeliazkov (Zheljazkov) Assistant Research Professor (Major Professor)	Dennis E. Rowe Research Professor (Committee Member)
_____	_____
Frank B. Matta Professor (Committee Member)	M. Wayne Ebelhar Professor (Committee Member)
_____	_____
William L. Kingery Professor and Graduate Coordinator of the Department of Plant and Soil Sciences	Melissa J. Mixon Interim Dean of the College of Agriculture and Life Sciences

Name: Vasile Cerven

Date of Degree: May 3, 2009

Institution: Mississippi State University

Major Field: Agriculture - Horticulture

Major Professor: Dr. Valtcho D. Jeliazkov (Zheljazkov)

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Candidate for degree of Master of Science

Peppermint dry weight biomass was higher in Verona (8119 kg/ha) than in Stoneville (6115 kg/ha). Overall both, oil content and yield were higher in bud formation stage than flowering stage. The levels of major essential oil constitutes were (-)-menthol 26 – 30 %, (-)-menthone 14 – 21 %, (+)-menthofuran 5 – 11 %, and eucalyptol 3 – 4 % of total essential oil content at flowering stage. Menthone content and its yield were higher at first cut; however, (+)-menthofuran content and its yield were higher at the second cut at bud formation. Although N fertilizers at rate 80 kg/ha did not affect essential oil content and yield at cut 1, N rate at 80 + 80 kg/ha increased oil yield at cut 2.

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CHAPTER I

INTRODUCTION

Plants produce many organic compounds whose function in growth and development is little understood. These substances are known as secondary metabolites, secondary products, or natural products (Taiz and Zeiger, 1998). The function of secondary metabolites is generally to protect plants against herbivores and pathogens. (Taiz and Zeiger, 1998). The aroma-yielding plants or their distilled volatile oils are used in various human activities, as active flavor and fragrant ingredients of perfumery and cosmetic concoctions, the demand for these oils are increasing in hygiene and health care formulations (Sangwan et al., 2001). Peppermint, *Mentha piperita* L. has economic importance because of its ability to produce and store essential oil, whose main constituent is menthol. Menthol is used in oral hygiene products, pharmaceuticals, cosmetics and the foods industries (Scavroni et al., 2005).

Mints are grown for the essential oil or for dried leaves. The United State has the largest cultivated area, with mint production in: Indiana, Michigan, Wisconsin, Oregon, Washington, Idaho, South Dakota, and Montana. Areas best suited to mint production are north of the 41st parallel where crops have 15 hours of daylight during the summer (Thomson et al., 1999). Oregon has the greatest peppermint production in the US and ranks fourth in spearmint production (Thomson et al., 1999). In Washington State,

approximately 9,720 ha of peppermint were harvested in 2002 (Ferguson and Walsh, 2002). In Montana, there were approximately 729 ha of spearmint and peppermint in 2002, which is 2.4 % of total US mint production (Petroff and Stougaard, 2002). Indiana is an important mint-producing state where peppermint production is 11.3 % of US mint production and there were four cultivar of peppermint: Black Mitcham, Todd's Mitcham, Murray Mitcham and Robert's Mitcham being grown in the state (Weller et al., 1998).

Many countries produce mint: US, Argentina, Australia, Brazil, Bulgaria, China, Egypt, France, Hungary, Japan, Korea, Morocco, New Zealand, Paraguay, Romania, Taiwan, and the UK. The production of peppermint oil in the world is near 4,000 tones $year^{-1}$ (Peterson and Bienvenu, 2009). Production of peppermint oil in the US in 2006 was estimated at 3291 tones (USDA, NASS, 2007).

The objective of this research was to investigate the potential of producing peppermint cv. Black Mitcham in Mississippi. The hypothesis for the research was that peppermint can be grown under Mississippi climatic conditions and its productivity and oil composition would be similar to peppermint grown in other regions of United States or other countries. There has been no previous published research on peppermint production in Mississippi. Thus, the main focus of this investigation was estimation of oil content at the different growth stages and determine qualitatively and quantitatively the oil composition of peppermint plants grown in Mississippi at two locations.

CHAPTER II

LITERATURE REVIEW

Terpenes are lipids synthesized from Acetyl CoA or from basic intermediates of glycolysis (Taiz and Zeiger, 1998). In the well-studied mevalonic acid pathway, three molecules of acetyl CoA are joined together stepwise to form mevalonic acid (Taiz and Zeiger, 1998). The monoterpenes are best known as the principal constituents of the essential oils and are produced by and stored in the peltate glandular trichomes (Turner et al., 2000).

The peppermint productivity and concentration range of the major essential oils constituents can vary depending on cultivar (Stoyanova et al., 2000), photoperiod (Clark and Menary, 1979 b). Scientists have studied different aspects of mint production. Examples of this research include the effect of heavy metals on peppermint and application of compost high in copper (Cu), cadmium (Cd), and lead (Pb) to peppermint (Zheljazkov and Nielsen, 1996; Zheljazkov and Warman, 2004); Zheljazkov et al., 2006), and sulfur influence on peppermint oil quality (Christensen et al., 2003). Much of the above research was aimed at improving sustainability of mint production (Maffei, 1999).

2.1 Nitrogen effect on mint productivity and essential oil

Environmental factors and agronomic practices influence essential oil quantity and quality. Indeed, fertilizers are important in mint crop production and many researchers are studying the effects of different fertilizers, different rates and harvesting management on yield and essential oil quality in different climates. Clark and Menary, (1980) reported significantly higher dry matter and essential oil yields for peppermint with higher N application. According to Brown et al. (2003) peppermint requires approximately 224 to 280 kg N ha^{-1} to support optimum growth. Multiple applications of N during the growing season are made to maintain a continuous nutrient supply for maximum oil production. The effect of irrigation and N on the yield and composition of peppermint oil was studied by Clark and Menary (1980). Peppermint was harvested twice during the growing season. Harvest first was conducted on 16 February 1979, and the subsequent regrowth was harvested on 25 April 1979. High peppermint oil yields were associated with increasing rates of N fertilizer (100, 200, and 300 kg N ha^{-1}), and high levels of irrigation (50 mm per week). The composition of oil extracted from the herb at the commercial harvest date (February 16 and April 25, 1979) was not significantly affected by N fertilization or irrigation treatments but the oil yield from regrowth within the same growing season was significantly affected by irrigation and by N treatments applied prior to the first harvest. The oil from regrowth contained high levels of menthol, menthyl acetate, menthofuran, limonene, and low levels of menthone and cineole (Clark and Menary, 1980).

The important role of N in regulating peppermint productivity and oil production is well-documented (Piccaglia et al., 1993, and Clark and Menary, 1980). Mitchell and

Farris, (1996) researched the optimum level of N application on Murray Mitcham peppermint in Central Oregon over two years, and found that a total (soil inorganic + fertilizer) N of around 280 kg ha^{-1} resulted in maximum oil yield and dry matter yields in arid climate conditions. The effect of N fertilization at (0, 60, 120, 180, 240 and 300 kg ha^{-1}) on the yield and chemical composition of the essential oil of peppermint cv. Black Mitcham grown on a Fox loamy-sand soil in Ontario Canada were determined during 1990 and 1991 by Court et al. (1993). Oil yields increased with increased N fertilization up to 180 kg ha^{-1}. Zheljazkov and Margina (1996) studied the application of increasing levels of N, phosphorus (P), and potassium (K) fertilizers to peppermint, and the results showed that the second highest essential oil component, after menthol, was menthyl acetate in all cultivars. Increasing the fertilization rate increased menthyl acetate content, without changing menthol content.

Composition of peppermint oil is affected by location. Oils produced in northern Tasmania had lower cineole and, in some cases, higher menthol than southern Tasmanian oils. Oil extracted from regrowth herbage after commercial harvest was distinguished by having very high menthyl acetate, low menthone, high menthol, high menthofuran, low cineole and low limonene concentrations (Clark and Menary, 1981). Research on productivity and biochemical composition of peppermint of different origins (Lithuanian, Polish and the cultivar Krasnodarskaya from Ukraine) grown under the same meteorological and agronomic conditions, indicated that their productivity and chemical value differed. Peppermint of Polish origin produced the highest green herb yield (10200-10700 kg ha^{-1}). In the raw biomass material of peppermint of Lithuanian origin there

were 0.17 % of essential oils, in cultivar Krasnodarskaya 0.42 %, and in peppermint of Polish origin the biggest amount – 0.60 % (Dambrauskiene et al., 2008).

The oil content and composition of two Bulgarian cultivars of peppermint obtained by hydrodistillation and dichloromethane extraction, respectively, were analyzed by GC/MS (Stoyanova et al., 2000). The oil content from cv. Zefir was 0.97 % and cv. Kliment– 63 was 0.54 %. The oil from cv. Zefir was found to be rich in menthol (46 – 50 %) and menthyl acetate (17 – 23 %). The oil from cv. Kliment – 63 had a lower content of these components, while the menthone content was higher (20 – 23 %).

2.2 Effect of harvesting time on mint productivity

Rohloff et al. (2005) researched the effect of harvest time and drying method on biomass production, essential oil yield and quality of peppermint and found that peppermint oil yield increased from early to full bloom and late bloom as an effect of biomass production and leaf growth. The flavor – impact compounds, menthol and menthone, reached their optimum at full bloom (43 – 54 % and 12 – 30 %, respectively).

Variation in the chemical composition of essential oil of peppermint during the growing season was measured for over three years by Chalchat et al., 1997. Level of major essential oil constitutes were dependent on harvesting time. Percentage of menthol and menthone harvested on June 14 were 22 % and 52 % respectively, however percentage of these oil constitutes harvested on October 30 were 53 % and 7 % respectively. The late blooming period gave oils rich in menthol and a second harvest gave high-quality oil and increased overall yield. Pre-drying did not affect the chemical

composition of the oil obtained but allowed larger amounts of plant material to be distilled.

The quantity and quality of essential oil depend on physiological stage of peppermint. According to Marotti et al. (1993) the biomass, oil and menthol content were greatest at the flowering stage. In addition, Clark and Menary, (1979 a) recorded that maximum oil yield and optimum oil quality was achieved by harvesting when the free menthol content of the oil exceeded 45 %, which occurred when plants had formed a terminal inflorescence about 1 to 2 cm in length.

Timing of harvest and other facets of management are important for both yield and quality of oil extracted from peppermint (Clark and Menary, 2006). The importance of harvest date and plant density on the yield and quality of Tasmanian peppermint oil was studied by Clark and Menary (1979 a). Oil yield of peppermint per unit area obtained from plant density treatments 30 and 40 plants m^{-2}, reached a maximum early in the growing season, whereas oil yield from the lower density treatment, 10 plants m^{-2}, continued to increase with menthol content of 50 %. The low density stand yielded less oil per unit area. Maximum oil yield and optimum oil quality was achieved by harvesting when the free menthol content of the oil had exceeded 45 %. As the growing season progressed, menthol and menthyl acetate contents of oil increased while menthone decreased. This effect was accelerated at the higher plant densities.

Effects of irrigation and harvest timing on peppermint oil yield in California were studied by Marcum and Hanson (2006). Highest oil yields of 118 kg ha^{-1} in 2003 and 119 kg ha^{-1} in 2004 occurred for harvest dates of July 29 and August 13, respectively. Menthol levels of 42 to 44 % were associated with the highest yields. Aflatuni et al.,

(2000) investigated variation in mints of different origins cultivated in Finland and reported that different harvesting times did not greatly affect the percentage of the main components of essential oils for any of the mints. At this stage (leaves were collected at an early flowering stage) of plant development, young leaves contained more menthone than menthol and the relative percentages for menthofuran and pulegone were low in the leaves.

The essential oil accumulation in peppermint is a complex phenomena and influenced not only by environmental factors such as light and temperature (Burbott and Loomis, 1967), but also agronomic factors such as fertilizers (Mustiatse, 1985; Alkire and Simon, 1995; Zheljazkov and Margina, 1996; Zheljazkov and Nielsen, 1996; Scavroni et al., 2005). Indeed nutrients play an important role in mint plant growth and development. Although N is not directly involved in the molecular structure of monoterpenes (menthone, menthol, menthofuran and eucalyptol), researchers (Clark and Menary, 1980 c; Singh et al., 1989; Piccaglia et al., 1993; Mitchell and Farris, 1996; Jeliazkova et al., 1999;) have shown that high peppermint oil yields were associated with high rates of N fertilization. Peppermint is produced on a wide range of soil types and climatic conditions. Hence, N requirements and its use efficiency is expected to vary for different soil types.

CHAPTER III

MATERIALS AND METHODS

3.1 Plant material and growing conditions.

The research was conducted during the 2007 cropping season at two locations in Mississippi: North Mississippi Research and Extension Center in Verona on Prentiss fine sandy loam soil and at Delta Research and Extension Center in Stoneville on Bosket very fine sandy loam soil. The experimental fields had been fallow for two years. Three soil samples were taken and analyzed for nutrient contents using the Lancaster soil test method (Cox, 2001) before land preparation. The concentrations of nutrients in soil extracts were measured with an inductively coupled argon plasma spectrometer in Soil Testing and Plant Analysis Laboratory in Mississippi State University. Before land preparation, phosphorus and potassium fertilizers were broadcast applied at rates of 79 kg P ha^{-1} and 103 kg K ha^{-1} with fertilizer spreader at Verona and Stoneville sites, respectively.

In early spring, the land was disked and raised beds (15 cm high and 75 cm wide) were formed using the Press pan bed shaper machine (Kennco Manufacturing, Inc., Ruskin, FL.). This machine also placed a drip-tape irrigation tube under the soil approximately 5 cm deep with the emitters facing upwards. Experimental plots were 6.1 m by 0.75 m with 40 plants planted two rows, with 30 cm between row spacing. The

sites were treated with herbicide Sinbar with 80 % terbacil as an active ingredient at 2 kg ha^{-1} and herbicide Roundup – 1.5 % on April 27, 2007. Half of the 32 plots were used for bud stage harvests and half were used for flower stage harvests. Virus free elite plant material was purchased from the Summit Plant Laboratories (3003 West Vine Drive, Fort Collins, CO): peppermint (*Mentha piperita* L.) cv. Black Mitcham. The seedlings were hand planted on May 4 and on May 8 - 9 in Verona and Stoneville, respectively.

During the vegetation period we did not observe powdery mildew on peppermint, but powdery mildew was observed on arvensis, which was close to peppermint experimental plots, so we sprayed all the mints, including peppermint, preventively with Quadris at 1037 ml ha^{-1} on July 26, 2007. However, peppermint was affected by Fusarium Stem Rot disease at the Delta Research and Experimental Station (Stoneville) on Bosket very fine sandy loam and we were not be able to take a second cut. The fungus is one of several soilborne pathogens that cause "non-specific stolon decline" in peppermint. Black cankers on the outside of the stem were typical symptom of the disease on many other types of plants (Balbalian, 2007). Plants infected by this fungus wilted and eventually died.

The experimental design was completely randomized design with four replicates. The N fertilizers (ammonium nitrate) were surface applied on experimental plots by hand. The treatments consisted of: (1) Control (without N); (2) 80 kg N/ha; (3) 80 kg N ha^{-1} + 80 kg N ha^{-1}. The first increment of 80 kg ha^{-1} of N was applied before transplanting, the second 80 kg ha^{-1} N was applied at bud formation stage harvest. Peppermint was harvested during bud formation and flowering stages by hand cutting plants at approximately 10-15 cm above ground: first peppermint harvest in bud

formation was on July 13 in Verona and July 17 in Stoneville and on October 2 the second harvest in bud formation in Verona; and first peppermint harvest at flowering stage was on August 4 in Verona and August 6 in Stoneville.

Subplots (91 cm by 75 cm) were harvested in each plot for essential oil extraction and dry weight calculations. The rest of each plot was harvested for total biomass plot's yield, except 30 cm at the end of the rows which was not harvested to avoid any edge row effect. The peppermint was kept from direct sunlight while air drying. The fresh and air dried peppermint plants' weights were recorded.

3.2 Essential Oil Extraction and Gas Chromatography-Mass Spectrophotometer Analyses

Essential oils were extracted from 250 g of air-dried plant material for 1 h (distillation time) by steam distillation, on modified Clevenger collector apparatus using a 2.0 L distillation system (Furnis et. al., 1989). The plant material was chopped into pieces approximately 1-3 cm in length prior to distillation. Peppermint essential oil from each treatment was weighed, and the oil yield was calculated as the weight (g) of oil per weight (g) of air-dried peppermint biomass. Chemical standards and peppermint oil from the field experiments were analyzed by Gas Chromatography-Mass Spectrophotometer Analyses (GC-MS) on a Varian CP-3800 GC coupled to a Varian Saturn 2000 MS/MS in USDA-ARS, University of Mississippi, Oxford (Zheljazkov et al., 2008). The GC was equipped with a DB-5 fused silica capillary column (30 m x 0.25 mm, with film thickness of 0.25 μm) operated using the following conditions: injector temperature, 240 ºC,

column temperature 60-240 ºC at 3 ºC min^{-1} then held at 240 ºC for 5 min; carrier gas, He; injection volume, (splitless) 1 μL (Zheljazkov et al., 2008)

3.3 Quantitative Analysis

Commercial standards for eucalyptol, (-)-menthol, (-)-menthone, and (+)-menthofuran, were purchased from Fluka (Switzerland). With five concentrations an external standard least squares regression for quantification was calculated. All four analytes were used to formulate separate calibration curves (Zheljazkov and Cantrell, 2009). All calculations were performed by generation of standard curves within Varian's (Palo Alto, CA) Saturn GC/MS Workstation software package Version 6.40 (Table 1). The chromatograms of each of the oils from the treated plants were compared to the standard injections. The target peaks were confirmed by both retention time and mass spectral data. Confirmed integrated peaks were then used to determine the percentage of each chemical constituent in the essential oil (Zheljazkov and Cantrell, 2009).

Table 1 Standard curve data of commercial standards run on Varian CP-3800 GC coupled to a Varian Saturn 2000 MS/MS*.

Standard	R^2 from Standard Curve	Retention Index[b]	Range (mg/mL)[c]	Limit of Quantitation (mg/mL)
(-)-menthol	0.999	1172	1.42-0.0142	0.00142
(-)-menthone	0.996	1153	0.535-0.0268	0.00268
(+)-menthofuran	0.965	1164	1.65-0.00165	0.00165
eucalyptol	0.999	1031	0.693-0.00346	0.00346

[a]All standards were commercially available. [b]Retention index computed as described previously by Kovats (Kovats, E. 1965. Pp. 229-247. In Advances in Chromatography, Vol. I., J.C. Giddings and R. A. Keller (eds.), Marcel Dekker, Inc. New York, NY.). [c] Range used for determination of R^2 and for quantitative analysis.

*With permission from Zheljazkov and Cantrell, 2009.

3.4 Statistical analysis

Analysis of variance and correlation analysis were executed with the PROC MIXED and PROC CORR programs, respectively (SAS Institute, 2003). The significance of N fertilization rate, and the location and harvests effects were determined at P = 0.05 level of significance.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Extractable soil nutrients of experimental soil types

Research was conducted on Prentiss fine sandy loam (http://ortho.ftw.nrcs.usda.gov/osd/dat/prentiss.html) at Verona, MS and Bosket very fine sandy loam (http://www2.ftw.nrcs.usda.gov/osd/dat/bosket.html) at Stoneville, MS. The concentrations of extractable soil nutrients (Table 2) show that soil at Stoneville had higher organic matter (1.37 %) than the soil at Verona (1.11 %), but both soil types contain relatively low organic matter. The soil pH was 6.0 at Stoneville to 6.1 at Verona, which was slightly acidic. The Verona soil had higher levels of (P) phosphorus (146 kg ha^{-1}) and (K) potassium (320 kg ha^{-1}) than Stoneville soil with P of 100 kg ha^{-1} and K of 230 kg ha^{-1} . In Prentiss soil, the calcium (Ca) and magnesium (Mg) are higher than in Bosket in 1.58 and 4.73 times, respectively. The amounts of zinc (Zn) and sodium (Na) were higher in Prentiss soil than in Bosket soil and the sulphur (S) concentrations were 179 and 221 kg ha^{-1} respectively in these soils. Cation exchange capacity in Prentiss soil was 11.44 and in Bosket soil was 7.79 milliquivalents (per 100 gm).

Table 2. Extractable nutrient levels in experimental soils*

			Extractable nutrients levels, kg ha^{-1}						
Location (Soil type)	Organic matter, %	pH*	P*	K*	Ca*	Mg*	Zn*	S*	Na*
Stoneville (Bosket very fine sandy loam)	1.37	6.1	100	230	2005	113	1.76	221	96
Verona (Prentiss fine sandy loam)	1.11	6	146	320	3172	535	3.95	179	103
* (Average of 3 samples, 0-15 cm)									

4.2 Effect of location, harvesting stage and N rate on peppermint biomass yield, oil content, oil composition and yield at flowering stage

The test of significant effects of location and N rate on peppermint biomass yield, oil and menthol content and yield at flowering stage are presented in Table 3. The location had a significant effect on peppermint dry weight biomass yield ($p = 0.01$). For example, the average Verona peppermint dry weight biomass was significantly higher (33 %) than production at Stoneville. Although N of 80 kg ha^{-1} did not significantly affect biomass yield, nevertheless it was consistently greater than the control (N0) at both locations.

The essential oil content and essential oil yield in peppermint at the flowering stage varied from 0.62 % to 1.12 % and from 47 kg ha^{-1} to 72 kg ha^{-1} (Table 3). This result indicates a great effect due to location on both the concentration and yield of

essential oil. The essential oil concentration at Stoneville was 177 % of that at Verona while the yield was 137 % greater.

Table 3. Effect of location and N rate on peppermint biomass yield, oil and (-)-menthol content and yield at flowering stage.

Location	N rate	Biomass Yield (kg ha^{-1})	Essential Oil		(-)-Menthol	
			Content, %	Yield, kg ha^{-1}	Content, %	Yield, kg ha^{-1}
Stoneville		6115 b*	1.10 a	66.8	28.8	19.0 a
Verona		8119 a	0.62 b	48.9	26.7	13.3 b
	0	6582	0.87	54.2	28.8	15.7
	80	7652	0.85	61.5	26.7	16.5
Stoneville	0	5482	1.12	61.5	29.8	18.2
Stoneville	80	6748	1.09	72.1	27.7	19.8
Verona	0	7681	0.62	46.9	27.7	13.3
Verona	80	8556	0.61	50.9	25.7	13.2
Analysis of variance		P values				
Location		**0.01**	**<0.01**	0.10	0.16	**0.04**
N rate		0.16	0.83	0.38	0.16	0.75
Location*N rate		0.79	0.91	0.69	0.99	0.73

* Means with the different letter within a column are significantly different at P = 0.05 level of significance.

The yield of essential oil was not significantly affected by location unlike biomass and concentration of essential oil. The locations had offsetting effects of greater concentration was associated with reduced yields and the effect was no location effect on oil yield.

The N fertilization of 80 kg ha^{-1} did not significantly affect essential oil concentrations in peppermint at either location. Since the concentrations at 0 and 80 kg N ha^{-1} were nearly identical, the effect of N on oil yield only reflected biomass differences which, as mentioned earlier, were not significantly affected by N fertilization. The concentrations of the major peppermint essential oil constitutes were: (-)-menthol 26 – 30 % (Table 3), (-)-menthone 14 – 21 %, (+)-menthofuran 5– 11 %, and eucalyptol 3 – 4 % (Table 4) of total essential oil at flowering stage.

Table 4. Effect of location and N rate on (-)-menthone, (+)-menthofuran and eucalyptol content and yield at flowering stage.

Location	N rate	(-)- Menthone		(+) - Menthofuran		Eucalyptol	
		Content, %	Yield, kg ha^{-1}	Content, %	Yield, kg ha^{-1}	Content, %	Yield, kg ha^{-1}
Stoneville		20.8 a*	13.8 a	10.7 a	7.2 a	4.4	6.5
Verona		14.2 b	7.2 b	5.7 b	3.0 b	3.1	6.1
	0	17.3	9.7	7.5	4.3	3.6	6.5
	80	17.7	11.2	8.9	5.9	3.9	6.1
Stoneville	0	21.0	12.9	9.6	5.9	4.0	6.5
Stoneville	80	20.6	14.7	11.7	8.5	4.7	6.5
Verona	0	13.6	6.5	5.3	2.6	3.1	6.5
Verona	80	14.9	7.8	6.1	3.4	3.1	5.7
Analysis of variance		P values					
Location		**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.09	0.19
N rate		0.78	0.38	0.06	0.10	0.62	0.16
Location*N rate		0.53	0.91	0.43	0.40	0.55	0.18

* Means with the different letter within a column are significantly different at P = 0.05 level of significance.

Peppermint essential oil composition was often significantly affected by location. The concentration of (-)-menthone and (+)-menthofuran at Stoneville were greater (20.8 % and 10.7 %), respectively than at Verona (Table 4) while concentrations of (-)-menthol (Table 3) and eucalyptol (Table 4) were not affected by location. None of the concentrations were significantly affected by N fertilization at the flowering stage (Table 3 and Table 4). The essential oil composition and its yield are important for the commercial production of this crop. These results indicated that the (-)-menthol, (Table 3), (-)-menthone and (+)-menthofuran yields (Table 4) were, respectively, 43 %, 92 % and 140 % greater at Stoneville than at Verona. Eucalyptol oil content and its yield were not significantly affected by locations at the flowering stage harvest (Table 4). Nitrogen fertilization of 80 kg ha^{-1} did not significantly affect dry biomass yield, oil content and oil yield at flowering stage (Table 3) either in Stoneville or in Verona. Effect of harvesting stage on peppermint oil content and (-)-menthol content and yield at cut 1 are presented in Tables 5.

Table 5. Effect of harvesting stage on peppermint oil and (-)-menthol content and yield at cut 1.

Location	Stage	N rate	Oil		(-)- Menthol	
			Content, %	Yield, kg ha^{-1}	Content, %	Yield, kg ha^{-1}
	Flowering		0.88 b*	57.5 b	27.8	16.0
	Bud formation		1.16 a	102.5 a	29.2	30.3
		0	1.05	79.9	29.3	24.0
		80	1.00	80.0	27.7	22.4
	Bud formation	0	1.23	105.6	29.8	32.2
	Bud formation	80	1.10	99.3	28.6	28.5
Stoneville			1.20 a	98.4 a	29.6	29.3
Verona			0.84 b	61.5 b	27.5	17.1
Stoneville	Flowering		1.10	66.8 b	28.8	19.0
Stoneville	Bud formation		1.30	130.0 a	30.3	39.5
Verona	Flowering		0.66	48.1 b	26.9	13.0
Verona	Bud formation		1.03	74.9 a	28.1	21.2
Stoneville		0	1.26	100.4	30.7	31.2
Stoneville		80	1.14	96.5	28.4	27.3
Verona		0	0.83	59.5	27.9	16.8
Verona		80	0.86	63.5	27.1	17.4
Analysis of variance			P values			
Stage			**<0.01**	**<0.01**	0.15	**<0.01**
N rate			0.28	0.99	0.11	0.32
Stage*N rate			0.11	0.21	0.74	0.19
Location			**<0.01**	**<0.01**	0.08	**<0.01**
Location*Stage			0.06	**<0.01**	0.87	**<0.01**
Location*N rate			0.11	0.44	0.41	0.18

* Means with the different letter within a column are significantly different at P = 0.05 level of significance.

Table 6. Effect of harvesting stage on peppermint oil and (-)-menthone, (+)-menthofuran content and yield at cut 1.

Location	Stage	N rate	(-) - Menthone		(+) - Menthofuran	
			Content, %	Yield, kg ha^{-1}	Content, %	Yield, kg ha^{-1}
	Flowering		17.0 a*	10.1 b	8.3	5.1 b
	Bud formation		22.7 b	25.5 a	8.3	8.3 a
		0	19.8	18.0	8.1	6.5
		80	20.0	17.5	8.6	6.9
	Bud formation	0	22.2	26.4	8.7	8.8
	Bud formation	80	23.3	24.6	7.9	7.9
Stoneville			25.0 a	26.0 a	9.4	9.0 a
Verona			14.7 b	9.6 b	7.3 b	4.5 b
Stoneville	Flowering		20.8 b	13.8 b	10.7 a	7.2
Stoneville	Bud formation		29.3 a	38.2 a	8.2 b	10.7
Verona	Flowering		13.2 b	6.3 b	6.0 b	3.0
Verona	Bud formation		16.2 a	12.8 a	8.5 a	6.0
Stoneville		0	25.4	27.2	8.8	8.6
Stoneville		80	24.7	24.8	10.0	9.4
Verona		0	14.1	8.9	7.4	4.5
Verona		80	15.3	10.3	7.1	4.5
Analysis of variance			P values			
Stage			**<0.01**	**<0.01**	0.98	**0.01**
N rate			0.89	0.81	0.63	0.67
Stage*N rate			0.64	0.56	0.19	0.13
Location			**<0.01**	**<0.01**	**0.03**	**<0.01**
Location*Stage			**<0.01**	**< 0. 01**	**0.01**	0.77
Location*N rate			0.60	0.37	0.43	0.64

* Means with the different letter within a column are significantly different at P=0.05 level of significance.

Table 7. Effect of harvesting stage on eucalyptol oil content and yield at cut 1.

Location	Stage	N rate	Eucalyptol	
			Content, %	Yield, kg ha^{-1}
	Flowering		6.4 b*	3.7 b
	Bud formation		7.2 a	7.3 a
		0	6.9	5.6
		80	6.7	5.5
	Bud formation	0	7.2	7.6
	Bud formation	80	7.1	7.1
Stoneville			6.9	6.9 a
Verona			6.6	4.2 b
Stoneville	Flowering		6.5	4.4 b
Stoneville	Bud formation		7.2	9.4 a
Verona	Flowering		6.2	3.1 b
Verona	Bud formation		7.1	5.3 a
Stoneville		0	6.9	7.0
Stoneville		80	6.9	6.7
Verona		0	6.9	4.1
Verona		80	6.4	4.2
Analysis of variance			P values	
Stage			**<0.01**	**<0.01**
N rate			0.27	0.74
Stage*N rate			0.49	0.33
Location			0.24	**0.01**
Location*Stage			0.66	**0.01**
Location*N rate			0.25	0.65

* Means with the different letter within a column are significantly different at P = 0.05 level of significance.

The results demonstrated that peppermint productivity depended on plant development stages. Both, oil concentration and oil yield were significantly overall higher in 30 % and 78 %, respectively, in bud formation stage than flowering stage (Table 5).

Peppermint essential oil content was dependent on locations. For example, essential oil concentration and yield were, respectively, 42 % and 60 % greater at Stoneville than Verona (Table 5). The concentration of (-)-menthone (Table 6) and eucalyptol (Table 7) were significantly higher 30 % and 12 %, respectively, at immature bud stage than in flowering stage. The plant development stages and the number of harvests per year were factors that influence peppermint oil yields, oil concentration and composition (Clark and Menary, 2006). The oil content of peppermint in the field experiment was higher when the plants were cut in the beginning rather than at the end of August. But, oil yield has been found to be higher when the plants were cut at the end of August due to higher herb yield and plant height (Aflatuni et al., 2006). Also, double cutting Black Mitcham peppermint enhanced essential oil yields by 200 % in 1994 and 54 % in 1995 as compared with single cutting (Welty et al., 1999).

4.3 Peppermint productivity affected by N rates at bud formation at cut 1 and cut 2

The peppermint dry weight biomass, oil and (-)-menthol content and yields at bud formation in Verona are presented in Tables 8. The total biomass yield and oil yield were significantly higher in 12 % and 17 % respectively at the first cut (July, 13) in comparison with the second cut (October, 2; Table 8). Essential oil concentration was not significantly influenced by cutting date in this research, but Telci and Sahbaz, (2005) reported that in Turkey the content was higher in the second harvest. The much higher peppermint oil yield at the first cut was due to the greater biomass production at first cut (Table 8). Overall, the first cut peppermint dry biomass yield (7543 kg ha^{-1}) was greater than the second cut (6703 kg ha^{-1}). Although there was no significant difference in oil

concentration between the cuttings dates (first cut 1.05 % and second cut 1.01 %) the magnitude of essential oil concentration at the second cut contributed overall 46 % to the total oil production of 146.6 kg/ha (79.1 + 67.5). The (-)-menthone concentration and its yield were significantly higher at first cut 3.85 and 4.44 times respectively in comparison with the second cut (Table 9). In contrast, the concentration of (+)-menthofuran and its yield were significantly higher at the second cut in 3.5 and 2.9 times, respectively, in comparison with the first cut (Table 9). Relatively to (+)-menthofuran, our results agree with the report of Ozal and Ozguven, (2002), who reported that the highest menthone + menthofuran levels were obtained from a third mint cutting. The eucalyptol yield was significantly higher by 24 % at first cut than the second cut (Table 10). This agrees with the results of Clark and Menary, (1980), who reported that peppermint oil from regrowth contained high level of menthofuran and low levels of menthone and cineole.

The menthol and menthone are the principal constituents of the peppermint essential oil (Zheljazkov and Margina, 1996; Stanev and Zheljazkov, 2004; Maffei and Mucciarelli, 2003). These compounds in our research were targeted by the cutting system and system of fertilization of peppermint in order to increase monoterpene alcohol such as (-)-menthol and decrease monoterpene ketones and oxides such as (-)-menthone and (+)-menthofuran. The commercial importance of peppermint essential oil depends on high percentage of (-)-menthol and (-)-menthone with concurrent lower percentages of (+)-menthofuran. The highest quality peppermint oils are characterized by a sweet odor and strong flavor, which is a result of high content of total and free menthol, and usually a low content of menthone (Shasany, et al. 2007). The magnitudes of these main

constituents in essential oil depended on level of mineral fertilization (Baird, 1957; Marotti et al., 2006). The peppermint dry weight biomass yields, oil and (-)-menthol content and yields at bud formation in Verona are presented in Table 8.

Table 8. Peppermint dry weight yields, oil and (-)-menthol content and yields at bud formation in Verona.

Cut date	N rate	Dry Weight biomass kg ha^{-1}	Oil		(-) - Menthol	
			Content, %	Yield, kg ha^{-1}	Content, %	Yield, kg ha^{-1}
Cut 1		7543 a*	1.05	79.1 a	28.8	22.9
Cut 2		6703 b	1.01	67.5 b	27.6	18.7
	0	6909	1.04	72.0	28.0	20.3
	80	7337	1.02	74.5	28.4	21.3
Cut 1	0	7083	1.09	77.1	29.6	22.9
Cut 1	80	8003	1.02	81.0	28.0	22.9
Cut 2	0	6735	1.00	67.0	26.4	17.7
Cut 2	80	6670	1.02	68.1	28.7	19.7
Analysis of variance		P values				
Cut date		**0.01**	0.56	**0.03**	0.42	0.05
N rate		0.17	0.71	0.62	0.83	0.63
Cut date*N rate		0.11	0.55	0.78	0.23	0.62

* Means with the different letter within a column are significantly different at P = 0.05 level of significance.

Table 9. Peppermint (-)-menthone, (+)-menthofuran content and oil yields at two harvests and two N fertilization rates at bud formation in Verona.

Cut date	N rate	(-) - Menthone		(+) - Menthofuran	
		Content, %	Yield, kg ha^{-1}	Content, %	Yield, kg ha^{-1}
Cut 1		22.7 a*	18.2 a	3.8 b	3.1 b
Cut 2		5.9 b	4.1 b	13.3 a	9.0 a
	0	14.6	11.3	8.8	6.0
	80	14.0	11.0	8.4	6.1
Cut 1	0	23.8	18.8	3.9	2.9
Cut 1	80	21.6	17.6	3.8	3.2
Cut 2	0	5.4	3.7	13.7	9.1
Cut 2	80	6.3	4.5	13.0	9.0
Analysis of variance		P values			
Cut date		**<0.01**	**<0.01**	**<0.01**	**<0.01**
N rate		0.69	0.90	0.59	0.92
Cut date*N rate		0.37	0.58	0.76	0.83

* Means with the different letter within a column are significantly different at P = 0.05 level of significance.

Table 10. Peppermint eucalyptol content and oil yield at two harvests and two N fertilization rates at bud phase in Verona.

Cut date	N rate	Eucalyptol	
		Content, %	Yield, kg ha^{-1}
Cut 1		7.3	5.7 a*
Cut 2		6.8	4.6 b
	0	7.2	5.2
	80	7.0	5.2
Cut 1	0	7.7	5.8
Cut 1	80	7.0	5.7
Cut 2	0	6.7	4.5
Cut 2	80	6.9	4.7
Analysis of variance		P values	
Cut date		0.11	**0.02**
N rate		0.46	0.96
Cut date*N rate		0.20	0.64

* Means with the different letter within a column are significantly different at P = 0.05 level of significance.

Table 11. Peppermint dry weight and oil yields as affected by N rates at bud phase in Verona.

Cut date	N rate	Dry weight biomass (kg ha^{-1})	Oil	
			Content, %	Yield, kg ha^{-1}
Cut 1	0	7083 b*	1.09	77.1
Cut 1	80	8003 a	1.01	81.0
Analysis of variance		P values		
N rate		**0.04**	0.46	0.56
Cut 2	0	6735 b	1.00	67.0 b
Cut 2	80	6670 b	1.02	68.1 b
Cut 2	80+80	8295 a	1.14	95.1 a
Analysis of variance		P values		
N rate		**<0.01**	0.21	**0.01**

* Means with the different letter within a column are significantly different at P = 0.05 level of significance.

Table 12. Peppermint (-)-menthol, (-)-menthone content, and oil yield as affected by N rates in bud phase in Verona.

Cut date	N rate	(-) - Menthol		(-) - Menthone	
		Content, %	Yield, kg ha-1	Content, %	Yield, kg ha^{-1}
Cut 1	0	29.6	22.9	23.8	18.8
Cut 1	80	28.6	22.9	21.6	17.6
Analysis of variance		P values			
N rate		0.44	0.98	0.38	0.61
Cut 2	0	26.4	17.7 b*	5.4 b	3.7 b
Cut 2	80	28.7	19.7 b	6.3 b	4.4 b
Cut 2	80+80	31.5	29.8 a	8.8 a	8.4 a
Analysis of variance		P values			
N rate		0.06	**0.01**	**0.04**	**0.01**

* Means with the different letter within a column are significantly different at P = 0.05 level of significance.

Table 13. Peppermint (+)-menthofuran, eucalyptol content and oil yields as affected by N rates in bud phase in Verona.

Effect	N rate	(+)-Menthofuran		Eucalyptol	
		Content, %	Yield, kg ha^{-1}	Content, %	Yield, kg ha^{-1}
Cut 1	0	3.9	2.9	7.7	5.8
Cut 1	80	3.8	3.2	7.0	5.7
Analysis of variance		P values			
N rate		0.64	0.57	0.16	0.75
Cut 2	0	13.7	9.1	6.7	4.5
Cut 2	80	13.0	9.0	6.9	4.7
Cut 2	80+80	15.4	14.9	6.3	6.0
Analysis of variance		P values			
N rate		0.35	0.08	0.29	0.06

Nitrogen fertilization at 80 kg ha^{-1} significantly increased dry peppermint biomass at the first cut during bud formation (8003 kg ha^{-1}) as compared with control (7083 kg ha^{-1}), Table 11). Moreover, increasing N level from 80 kg ha^{-1} to 160 kg ha^{-1}, significantly increased peppermint dry biomass at second cut (8295 kg ha^{-1}) when harvested during bud formation in Verona (Table 11). Results indicated that effectiveness of N fertilization rate was dependent on number of mint cutting (harvesting) and second fertilization which was applied after the first harvest. Particularly, the yield for N fertilization at 80 kg ha^{-1} at first cut was 920 kg ha^{-1} in comparison with control, but at the second cut the same rate of N (80 kg ha^{-1}) did not significantly affect peppermint dry biomass in bud stage. Moreover, its dry biomass tended to decrease with the N rate at 80 kg ha^{-1}. These show that the N rate 80 kg ha^{-1} may not be enough for the whole season as would be expected. These results suggest the N rate 80 kg ha^{-1} may not sustain production for the whole

season. The best-yielding treatment for peppermint cv. Murray in central Oregon was N rate at 280 kg N ha^{-1} applied three times as a split application in the spring (Mitchell and Farris, 1994). Furthermore, oil production and its quality at cut 1 and cut 2 depended on N fertilization. In fact, N fertilizers at rate 80 kg ha^{-1} did not affect essential oil concentration and its yield at cut 1, N rate at 160 kg ha^{-1} significantly increased oil yield in 42 % with comparison with control at cut 2 (Table 11). Essential oil composition, was not significantly affected by N rate at 80 kg ha^{-1} at cut 1 and cut 2, but (-)-menthol yield, (-)-menthone content and yield were significantly increased respectively in 68 %, 63% and 127 % by N level 160 kg ha^{-1} in comparison with control at cut 2 (Table 12). These results show the need for more additional fertilizer N following the first cut and probably would be important for additional cuts.

4.4 Correlation coefficients for dry weight, essential oil content, and composition

Pearson's correlation coefficients, which measure on average, how the values of two variables are associated, are presented in tables 14. The results show that concentrations of eucalyptol, and (-)-menthol are positively correlated with oil content and oil yield, and (-)-menthone correlated with peppermint oil yield (Table 14). The oil yield was positively correlated with dry weight and concentration of essential oil in peppermint (Table 15). In addition, eucalyptol, (-)-menthone, and (-)-menthol yields were significantly positively correlated with peppermint's dry weight, concentration of eucalyptol, essential oil and its yield. However, the (+)-menthofuran yield was correlated only with the concentration of essential oil and its yield (Table 15).

Table 14. Correlations and their P values statistical significances between yield, oil content and oil yield by eucalyptol, (-)-menthone, (-)-menthol and (+)-menthofuran contents

	Oil	Eucalyptol	(-)-Menthone	(-)-Menthol	(+)-Menthofuran
	Content, %	Content, %	Content, %	Content, %	Content, %
Dry weight	0.13	0.09	0.46	0.17	-0.23
P values	0.66	0.75	0.12	0.58	0.45
Oil, content, %		0.69	0.47	0.80	0.34
P values		**0.01**	0.12	**<0.01**	0.27
Oil yield		0.59	0.6	0.71	0.12
P values		**0.04**	**0.03**	**0.01**	0.70

Table 15. Correlations and their P values statistical significances between yield, oil content and oil yield by eucalyptol, menthone, menthol and menthofuran yields.

	Oil	Eucalyptol	Menthone	Menthol	Menthofuran
	Yield	Yield	Yield	Yield	Yield
Dry weight	0.65	0.63	0.65	0.62	0.25
P values	**0.02**	**0.02**	**0.02**	**0.02**	0.41
Oil content, %	0.83	0.83	0.67	0.84	0.67
P values	**<0.01**	**<0.01**	**0.01**	**<0.01**	**0.01**
Oil yield		0.99	0.87	0.99	0.65
P values		**<0.01**	**<0.01**	**<0.01**	**0.02**
Eucalyptol content, %		0.67	0.58	0.59	0.15
P values		**0.01**	**0.04**	**0.04**	0.63

A correlation analysis between the essential oil constituents (Table 16) show that concentrations of eucalyptol, and (-)-menthone were positively correlated with yields of eucalyptol, (-)-menthone, (-)-menthol; (-)-menthol concentration with yields of eucalyptol, (-)-menthol, and (+)-menthofuran. Menthofuran content was positively correlated only with menthofuran yield. Furthermore, (-)-menthone yield were positively correlated with eucalyptol yield, (-)-menthol yield with eucalyptol and (-)-menthone yields and (+)-menthofuran yield with eucalyptol and (-)-menthol yields (Table 17). A very close positive correlations were observed between concentrations of (-)-menthol and oil, (-)-menthol yield and oil yield (-)-menthol yield and eucalyptol yield.

Table 16. Correlations and their P values statistical significances overall for all measurements: oil content

	Eucalyptol	Menthone	Menthol	Menthofuran
	Content, %	Content, %	Content, %	Content, %
Menthone %	0.48		0.38	-0.53
P values	0.10		0.22	0.07
Menthol %	0.56	0.38		0.23
P values	0.05	0.22		0.46
Menthofuran %	-0.13	-0.53	0.23	
P values	0.66	0.07	0.46	
Eucalyptol yield	0.67	0.64	0.70	0.04
P values	**0.01**	**0.02**	**0.01**	0.88
Menthone yield	0.58	0.89	0.54	-0.27
P values	**0.04**	**<0.01**	0.06	0.39
Menthol yield	0.59	0.58	0.77	0.13
P values	**0.04**	**0.04**	**0.01**	0.68
Menthofuran yield	0.15	-0.08	0.59	0.81
P values	0.63	0.79	**0.04**	**0.01**

Table 17. Correlations and their P values statistical significances overall for all measurements: oil yields

	Menthone	Menthol	Menthofuran
	Yield	Yield	Yield
Eucalyptol yield	0.90	0.98	0.57
P values	**<0.01**	**<0.01**	**0.04**
Menthone yield		0.86	0.27
P values		**0.01**	0.38
Menthol yield			0.66
P values			**0.01**

CHAPTER V

SUMMARY AND CONCLUSION

This experiment was conducted in 2007 and measured the effects of N rates, location, and harvesting stage on peppermint productivity, oil content, and oil composition under Mississippi growing conditions. Results showed that peppermint dry weight biomass was significantly higher in Verona on Prentiss fine sandy loam soil (8119 kg ha^{-1}) than in Stoneville on Bosket very fine sandy loam soil (6115 kg ha^{-1}) at flowering stage. Essential oil concentration in peppermint biomass was significantly higher in Stoneville (1.10 %) than in Verona (0.62 %). The levels of major peppermint essential oil constituents were: (-)-menthol 26 – 30 %, (-)-menthone 14 – 21 %, (+)-menthofuran 5 – 11 %, and eucalyptol 3 – 4 % of total essential oil content at flowering stage. The concentrations of (-)-menthone and (+)-menthofuran were significantly higher (20.8 % and 10.7 %) at Stoneville than in Verona (14.2 % and 5.7 %), respectively. The results also showed that the (-)-menthol, (-)-menthone and (+)-menthofuran oil yields were significantly higher in Stoneville (in 43 %, 92 %, and 140 %, respectively) than in Verona at flowering stage. Timing of peppermint's harvest is important for both yield and its quality because quantity and quality of essential oil depend on development stages of peppermint. Overall, both the oil concentration and oil yield were significantly higher (32 % and 78 %, respectively) in bud formation stage than in flowering stage. The yields of

(-)-menthone (152 %), (+)-menthofuran (63 %), and eucalyptol (97 %) were significantly higher in bud formation than in flowering stage. The harvest and number of harvests per year are factors that influenced peppermint oil yields, oil concentration and composition. The oil yield was significantly greater (17 %) at first cut than the second cut at bud formation. Menthone concentration and yield were higher at first cut respectively 285 % and 344% in comparison with the second cut. However, (+)-menthofuran concentration and its yield were significantly higher at the second cut respectively 250 % and 190 % in comparison with the first cut. The eucalyptol yield was significantly higher (24 %) at first cut in comparison with the second cut. Furthermore, oil production and its quality at cut 1 and cut 2 were dependent on N rate. Although N fertilization at 80 kg ha^{-1} did not affect essential oil concentration and yield at cut 1, N rate at 80+80 kg ha^{-1} significantly increased oil yield (42 %) at cut 2. Essential oil composition was not significantly affected by N rate at 80 kg ha^{-1} at cut 1 and cut 2, but (-)-menthol yield and (-)-menthone concentration and yield were significantly increased respectively in 68 %, 63 % and 127 % in comparison with control at cut 2. The results showed that concentrations of eucalyptol, and (-)-menthol were positively correlated with oil content and oil yield, and (-)-menthone correlated with peppermint oil yield. The oil yield was positively correlated with dry weight and concentration of essential oil in peppermint. In addition, eucalyptol, (-)-menthone, and (-)-menthol yields were significantly positively correlated with peppermint's dry weight, concentration of eucalyptol, essential oil and its yield. However, the (+)-menthofuran yield was correlated only with the essential oil concentration and yield. The concentrations of eucalyptol, and (-)-menthone were positively correlated with yields of eucalyptol, (-)-menthone, (-)-menthol; (-)-menthol

concentration with yields of eucalyptol, (-)-menthol, and (+)-menthofuran. Menthofuran content was positively correlated only with menthofuran yield. Furthermore, (-)-menthone yield were positively correlated with eucalyptol yield, (-)-menthol yield with eucalyptol and (-)-menthone yields and (+)-menthofuran yield with eucalyptol and (-)-menthol yields.

Although the present study gives a significant amount of information about peppermint dry biomass, essential oil yield and its composition depending on locations, number of cutting per year, harvesting stages, and N fertilizer, still much remains to be studied. Neither the optimum N fertilization rate or the necessity of split applications effects on essential oil quality, which is important to industry, has been fully researched. Irrigation and other factors may impact Fusarium Stem Rot (Fusarium species) disease which could negatively impact mint cultivation. Further research with well-developed designs are needed to determine the potential evaluate mint as a new crop for Mississippi.

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