

**EFFECTS OF NITROGEN RATE AND PLANTING DATE ON GROWTH,
YIELD AND CHEMICAL COMPOSITION OF MALABAR SPINACH
(*BASELLA ALBA* L.) UNDER CANADIAN MARITIME CLIMATIC
CONDITIONS**

by

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*This research work is dedicated to
my parents and to my husband*

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ABSTRACT

Malabar spinach (*Basella alba* L.) is one of the ethnic leafy vegetables in Canada, which is popular among immigrants of Asian and African origins. However, there are no clearly established production practices for commercial production of Malabar spinach in Canada. Therefore, this research study was conducted to determine the effects of nitrogen (N) rate and planting date on growth, yield and chemical composition of Malabar spinach under Canadian maritime climatic conditions. Our results showed 80 kg N ha⁻¹ is optimal for the greenhouse production of Malabar spinach. Field experiment results indicated 40 kg N ha⁻¹ is optimal for early planting. For mid and late plantings there were no significant differences in the growth and yield of the control and the N applied treatments. Nitrogen rate and planting date had little effect on phenolic and carotenoid content. Nitrate content was found very low regardless of N application rate.

LIST OF ABBREVIATIONS USED

NH ₄ ⁺	Ammonium ion
ACI	Anthocyanin content index
C	Carbon
CO ₂	Carbon dioxide
cm	Centimetre
DAS	Days after sowing
°C	Degree Celsius
DW	Dry weight
FW	Fresh weight
g	Gram
h	Hour
h d ⁻¹	Hour per day
LA	Leaf area
μL	Microliter
mg	Milligram
mL	Millilitre
mm	Millimetre
min	Minute
nm	Nanometre
NO ₃ ⁻	Nitrate
NO ₂ ⁻	Nitrite
N	Nitrogen
N ₂	Nitrogen gas
N ₂ O	Nitrous oxide
P ₂ O ₅	Phosphorus pentoxide
K ₂ O	Potassium oxide
rpm	Revolutions per minute
s	Second
SPAD	Soil plant analysis development
SLA	Specific leaf area
cm ²	Square centimetre
m ²	Square meter
mm ²	Square millimetre
TPC	Total phenolic content
TCC	Total carotenoid content
UV/VIS	Ultraviolet / Visible light
(CO(NH ₂) ₂)	Urea
v/v	Volume /volume
w/v	Weight in volume

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CHAPTER 1. INTRODUCTION

1.1. Introduction

Canada is one of the most multicultural and diverse countries in the world due to the proportionally large immigrant population. Statistically, Canada has the highest per capita immigration rate in the world, which will not diminish soon as more potential applicants are meeting the requirements of many different immigration programs (Statistics Canada 2017). The assurance of and access to culturally appropriate food are major determinants of food security for most of these immigrants for comfort, and their psychosocial and economic well-being. Therefore, ethnic food crops are gaining importance in vegetable markets of Canada.

A detailed study conducted on the preferences of ethnic vegetables by an immigrant population of Afro-Caribbean origin in the Greater Toronto Area gives a list of 13 vegetables that are of high sociocultural importance among immigrants (Adekunle et al. 2011). Despite the high interests and need, there are many challenges that need to be overcome for the successful production of ethnic vegetables in Canada. These include crop management practices, influence of climate, soil types, local pests and diseases (Filotas 2009). More importantly, it is unknown how climatic and edaphic factors will influence accumulation of phytochemicals (nutritional and anti-nutritional factors) in edible plant tissues and the extent of their effects on human health.

Preliminary trials were conducted at Dalhousie University's Faculty of Agriculture experimental site located at Bible Hill, Nova Scotia, Canada (45°23' N, 63°14' W). Trials to evaluate productivity of different tropical ethnic vegetables including okra

(*Abelmoschus esculentus*), eggplant (*Solanum melogena*), cassava (*Manihot esculenta*), amaranth (*Amaranthus tricolor*), Malabar spinach (*Basella alba*) and cowpea (*Vigna unguiculata*) during summer 2017 showed positive results. The growth and quality performance of Malabar spinach as shown in the Figure 1.1 was most promising. However, further studies on agronomy and post-harvest quality in response to management practices and environmental conditions are required prior to recommendations of these crops for their incorporation into local agricultural systems. This project seeks to find answers to some of these questions raised for the successful production of tropical ethnic vegetables in Canada.



Figure 1.1. Malabar spinach (*Basella alba* L.).

1.2. Research Hypothesis and Objectives

Hypothesis

Although Malabar spinach is a non-traditional crop in Canada, it has potential to grow commercially under maritime climatic conditions. The interactions of N rate and planting date can influence the growth, yield and chemical composition of Malabar spinach.

Overall Objective:

To evaluate the production potential of ethnic leafy vegetable Malabar spinach under Canadian maritime climatic conditions.

Specific Objectives are:

- 1) to study the effect of nitrogen rate on growth and yield of Malabar spinach under controlled environmental conditions; and
- 2) to evaluate the effects of nitrogen rate and planting date on growth, yield and chemical composition of Malabar spinach under field conditions.

1.3. Thesis Organization

This thesis consists of five chapters including the current chapter (Introduction). Chapter 2 is the literature review, and this chapter reviews the relevant literature done in the field of study. Chapter 3 concentrates on detailed explanation of materials and methods used in this study. Chapters 4 and 5 present results and discussion of the research, respectively. The thesis concludes with recommendations in Chapter 6 followed by the combined references for all the chapters.

CHAPTER 2. LITERATURE REVIEW

2.1. Importance of Vegetables in Human Nutrition

A worldwide survey on vegetables showed that at least 402 vegetables representing 69 families and 230 genera are under commercial cultivation (da Silva Dias and Imai 2017). Among these plants, about 53% represent leafy vegetables followed by 15% for fruit vegetables and 17% for roots and tuber vegetables (da Silva Dias and Imai 2017). Vegetables are humankind's most affordable source of vitamins and minerals needed for good health. Earlier, it was believed that there were 14 vitamins and 16 minerals that were essential for human health and wellbeing (da Silva Dias and Imai 2017). However, with recent scientific development in functional food and nutrition, there are many other phytochemicals present in vegetables that are essential and more beneficial than just vitamins and minerals to human health (da Silva Dias and Imai 2017).

The quality of vegetables is attributable to their high levels of vitamins, antioxidants, carotenoids, flavonoids and micronutrients which are naturally occurring (Fulton et al. 2016). Dietary intake of phytochemicals is inversely associated with the risk of cardiovascular diseases and several chronic ailments (Rodriguez-Casado 2016). Green leafy vegetables are rich sources of carotenoids which help to alleviate vitamin A deficiency and age-related macular degeneration, which are considered as serious public health concerns among children and adults in many developing countries (Raju et al. 2007). Thus, vegetables are designated as protective foods (Alissa and Ferns 2017). A new dietary pattern called MIND (Mediterranean-Dietary Approaches to Stop Hypertension (DASH) Intervention for Neurodegenerative Delay) has been developed at Rush University, Chicago, to avoid the risk of cognitive decline with age.

MIND emphasizes intake of natural plant-based foods, especially green leafy vegetables, and limited intake of animal and high saturated fat foods (Richardson 2017).

2.2. Ethnic Vegetables

Generally, ethnic vegetables refer to those non-traditional crops that are new to a region or a grower, and production is under low acreages and targeted to niche markets (Filotas 2009). It is also defined as those vegetables that are consumed by a group sharing the same cultural heritage (Adekunle et al. 2012). Okra (*Abelmoschus esculentus*), eggplant (*Solanum melogena*), amaranth (*Amaranthus tricolor*), vegetable soybeans (*Glycine max*), artichoke (*Cynara cardunculus* var *scolymus*), bitter melon/gourd (*Momordica charantia*) and many more crucifers, legumes and cucurbits are some of the ethnic vegetables being grown in Ontario (Cerkauskas et al. 1998; Hein 2014). These vegetables were almost unknown in Canada 50 years ago but are now growing in many provinces to meet immigrants' demand.

Ethnic vegetables are grown in home gardens, co-operative farms, market gardens, community supported agriculture farms and community gardens by immigrants (Whitehead et al. 2002; Wakefield et al. 2007). Growing ethnic vegetables locally helps to increase access to culturally appropriate vegetables at an affordable price and contribute positively to social cohesion with therapeutic horticultural benefits (Wakefield et al. 2007). Most of the ethnic vegetables are of tropical origin, hence the major constraint faced by growers in Canada is the availability of warm weather conditions and long frost-free period. However, warm summer months provide great opportunity to grow these vegetables successfully (Fruit and vegetable 2016).

2.3. Origin and Distribution of Malabar Spinach

Malabar spinach (*Basella alba* L., $2n = 48$) belongs to the family Basellaceae and is a perennial vine cultivated extensively in tropical Asia and Africa as a green leafy vegetable (Roy et al. 2010). It is known under names such as Malabar spinach, Indian spinach, vine spinach and Ceylon spinach (Roy et al. 2010). Malabar spinach is considered native to tropical Asia (Reddy et al. 2014). The distribution of Malabar spinach extends from tropical to subtropical zones of the world. It is now widely cultivated and naturalized in the tropics and is even grown in temperate regions as an annual plant (Abukutsa-Onyango 2004).

2.4. Botany and Cultivation of Malabar Spinach

Malabar spinach is a perennial twining herb. The stem is long, slender, succulent, glabrous and much branched (Deshmukh and Gaikwad 2014). There are two important species (*Basella alba* and *Basella rubra*) of Malabar spinach that are differentiated by their leaf characteristics and stem colors. The stem, petioles and leaves of *B. alba* are green whereas, in the species *B. rubra* the stem, petioles and leaves are red to violet in color (Kumorkiewicz and Wybraniec 2017). Leaves are thick, alternate, broad, heart shaped, short petiolate and tapering to a pointed tip with mucilaginous textured stems and leaves (Palada and Crossman 1999). The inflorescence of this plant is a hanging axillary spike with long peduncle and bisexual flowers (Abukutsa-Onyango 2004). Bracteoles are acute and the ovary is unilocular (Deshmukh and Gaikwad 2014). Stamens consist of short filaments and cordate anthers (Deshmukh and Gaikwad 2014). Fruit is purplish black coloured that contains violet juice and one seed. Seeds exhibit epigeal germination (Abukutsa-Onyango 2004).

Malabar spinach performs well in tropical and subtropical climates mainly because it is a warm season crop and extremely heat tolerant (Reddy et al. 2014). The plant is adaptable to a wide range of soils, but it is quite responsive to nitrogen fertilizer (Reddy et al. 2014). The optimal temperature for plant growth range is 20-35°C, and it is a short-day plant (AVRDC 2003). Flowering is inhibited at day lengths longer than 13 h (Abukutsa-Onyango 2004). The plant exhibits C₄ photosynthesis that leads to increased photosynthesis and high dry matter content under high light, temperature and adequate moisture and soil fertility (Abukutsa-Onyango 2004). Plants can be propagated by seeds and stem cuttings while seed soaking in water is recommended for germination (Acikgoz and Adiloglu 2018).

2.5. Importance of Nitrogen Fertilization in Crop Production

Nitrogen (N) is one of the essential plant nutrients, which occupies a unique position because of its abundant requirement by plants compared to the other essential nutrients (Hofman and Van Cleemput 2004). Nitrogen is a key element that regulates various plant metabolic functions. It is an important constituent of chlorophyll, nucleic acids and amino acids, all of which have crucial roles in plant growth and development (Rajasekar et al. 2017). Nitrogen can be absorbed and utilized by plants in two distinct inorganic forms i.e. either as nitrate (NO₃⁻) or ammonium (NH₄⁺). Unfortunately, N is universally deficient in almost all the agricultural soils and cropping systems around the world (Yadav et al. 2017). This is due to the dynamic nature of the N-cycle within the soil-plant systems. Therefore, N is considered as the most critical externally added input in any cropping system (Yadav et al. 2017).

Under natural conditions, N enters the soil ecosystem either through biological N fixation and/or decomposition of animal or plant residues (Below 2001). Biological N fixation is a natural process that involves the conversion of an unavailable form of nitrogen gas (N_2) from the atmosphere into the plant available form by certain soil microorganisms and leguminous plants (Hofman and Van Cleemput 2004). Although more than 90% of the N in soils is contained in the form of organic matter derived from decomposed animal and plant residues, plants cannot use this organic N unless it is transformed into plant available forms through the process of mineralization (Below 2001). Through the mineralization process, organic forms of N such as proteins, amino acids, purines and pyrimidines get transformed into plant usable inorganic form of N (i.e. NO_3^- and NH_4^+) with the help of potential soil microorganisms (Yadav et al. 2017). However, both biological N fixation and soil mineralization have their own constraints that limit the availability of N to the plants (Yadav et al. 2017). Nitrogen availability through biological N fixation is not enough to satisfy specific crop N demand while soil mineralization process is too slow and only 2-3% of the N is converted to available forms per year (Below 2001). In addition, soil mineralization is significantly affected by soil management practices and environmental conditions. As a result, addition of N from synthetic chemical fertilizers is usually required to meet crop demand (Below 2001).

2.6. Effects of Nitrogen Over Fertilization

Nitrogen fertilization is associated with dark-green coloration of leaves, increased vegetative growth, and enhancement of crop yield (Rajasekar et al. 2017). Hence, N application is important to achieve high crop yield and good quality horticultural produce (Tremblay and Bélec 2006). Unfortunately, some farmers indiscriminately

apply high N fertilizer for vegetable production with an intent of obtaining higher yields (Alborzon 2016). Excessive use of N in order to obtain high yields causes adverse effects on soil health, environment and quality of the agriculture produce (Tremblay and Bélec 2006). Therefore, N fertilizer management is one of the key challenges faced by vegetable growers.

Heavy N fertilization of fields leads to various environmental problems such as ground water contamination, release of greenhouse gas nitrous oxide (N_2O) and eutrophication of aquatic ecosystems (Albornoz 2016). Nitrogen fertilizer applied above the optimal cannot be absorbed and utilized by plants but will remain as residual soil mineral N after harvest (Tremblay and Bélec 2006). Residual soil N is very susceptible to external environment regardless of any form of N fertilizer. Nitrate-N is very mobile in nature and can be lost through leaching processes such as heavy rainfall or over irrigated conditions (Yadav et al. 2017). Nitrate-N can move beyond the soil profile to contaminate groundwater. On the other hand, NH_4^+ -N fertilizers like urea are more stable when applied through broadcasting. Under less favorable conditions, NH_4^+ -N fertilizers can cause ammonia volatilization, a process of conversion of NH_4^+ -N into NH_3 gas that escapes to the atmosphere (Yadav et al. 2017). Additionally, N fertilizers applied at non-optimal levels are the potential sources for the formation of greenhouse gas N_2O (Tremblay and Bélec 2006).

Excessive N application also affects the quality of vegetables as described in the Figure 2.1 (Albornoz 2016). High N application reduces the concentrations of vitamin C and phenolic compounds in many vegetables like spinach (*Spinacia oleracea*), tomato (*Solanum lycopersicum*) and lettuce (*Lactuca sativa*). This is due to a shift in plant

metabolism towards more N-containing compounds (proteins) than carbon-containing compounds such as sugars (Rembialkowska 2007; Albornoz 2016). High N application reduces dietary fiber and dry matter content of vegetables such as white cabbage (*Brassica oleracea*), carrot (*Daucus sativus*), broccoli (*Brassica oleracea* var *italica*) and leek (*Allium ampeloprasum*) which is an important qualitative factor for the processing industry (Sorensen 1999). More importantly, excessive N rate accumulates NO_3^- in plant leaves, which can be toxic for human consumption (Albornoz 2016; Kosson et al. 2017). Hence, optimum rate of N application is crucial in vegetable production to achieve high quality harvested produce.

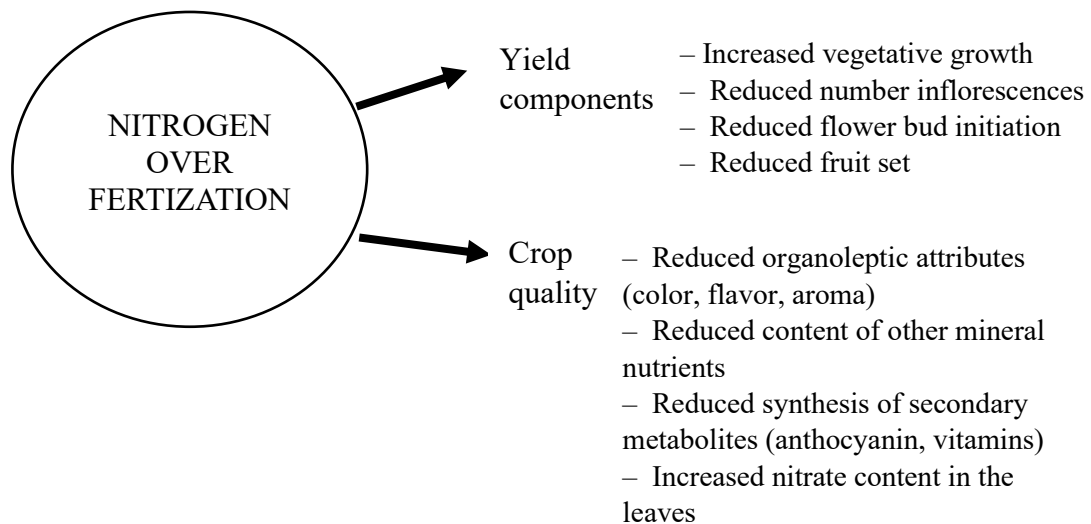


Figure 2.1. Effect of high nitrogen application on yield and quality of vegetables.

2.7. Nitrate - as an Antinutrient Factor

Nitrate accumulation in plant cells is affected by genetic, agronomic and environmental factors. Agronomic factors include application rate, timing and form of N fertilizer applied. Also, environmental factors like light intensity, temperature, photoperiod and carbon dioxide concentration play important roles in NO_3^- accumulation (Colonna et al. 2016). However, genetic background of the crop, amount and form of N application

and light intensities during crop growing season predominantly influence NO_3^- accumulation in fresh vegetables (Colonna et al. 2016).

Plants accumulate NO_3^- in the leaves when plant N uptake from the soil exceeds metabolic needs (Blom-Zandstra 1989). As such, excessive application of N fertilizer in vegetable production is more of a concern because of its detrimental impact on human health. Nitrate alone is relatively non-toxic. However, it can act as a reservoir to produce nitrite (NO_2^-) via a process known as enterosalivary circulation by bacterial action within the body during digestion (McMullen et al. 2005). Reduction of NO_3^- to NO_2^- leads to the formation of carcinogenic *N*-nitroso compounds caused by the reaction with secondary and tertiary amines present in the body. These compounds can readily react with haemoglobin; the haemoglobin is oxidized to methaemoglobin to cause blue baby syndrome or methemoglobinemia in infants and adults (McMullen et al. 2005). Therefore, optimum and judicious application of N fertilizers is very important in vegetable crop production.

To avoid excessive intake of nitrate in the diet, The Joint Food and Agricultural Organisation/World Health Organisation has set the acceptable daily intake (ADI) for NO_3^- at 3.7 mg kg^{-1} body weight (Hord and Conley 2017). This is because many research studies have revealed that vegetables, particularly leafy vegetables are the major source for dietary intake of NO_3^- . The European Commission regulation also established the thresholds of NO_3^- in spinach (2500-3500 mg kg^{-1} FW), lettuce (3000-5000 mg kg^{-1} FW), lettuce type 'Iceberg' (2000-2500 mg kg^{-1} FW) and for rocket (*Eruca vesicaria* ssp. *sativa*) (6000-7000 mg kg^{-1} FW) (Colonna et al. 2016).

2.8. Effects of Seasonal Variations on Crop Production

Changing environmental conditions like rainfall, temperature, day length and light intensity have strong effects on crop growth, yield and quality (Weston and Barth 1997). Uptake of available soil mineral nutrients by plants are largely influenced by external air and soil temperatures (Pregitzer and King 2005). At low temperatures, plants gradually lose their ability to absorb nutrients. It was earlier shown that at low temperatures, the pattern of plant nutrient uptake was $N > P > Ca > S > K$ (Zhurbitzky and Shtrausberg 1958). Frota and Tucker (1972) demonstrated that the absorption of NO_3^- and NH_4^+ increased with increasing temperature from 8°C to 23°C.

A study conducted in Mozambique on vegetative growth of *Amaranthus hybridus* and *A. tricolor* under different seasons showed that the effect of temperature on leaf yield was greatly determined by the length of day for both species (Ribeiro et al. 2017). It was further stated that temperature had significant positive effect on leaf yield, which was greater for longer day lengths (Ribeiro et al. 2017). Another study conducted by Whitehead et al. (2002) on *A. tricolor* in the USA reported that different planting date during the crop growing season had significant effect on plant growth and leaf yield due to temperature and day length. High soil temperatures during warmest part of the summer helped in good establishment of seedlings (Whitehead et al. 2002). Also, differences between day/night temperatures and photoperiod regulate flower initiation in most of the vegetables (Weston and Barth 1997).

Numerous literature studies have also revealed that the postharvest nutritional quality of fresh vegetables is generally determined by preharvest growing conditions. Yoon et al. (2017) reported significant increase of soluble sugar, vitamin C and amino acids in

spinach during cold stress. Concentrations of phenolics and flavonoids have significantly increased in leafy vegetable lettuce by increasing temperature and radiation during the growing season (Marin et al. 2015). Synthesis and accumulation of anthocyanins in vegetables are very sensitive to environmental conditions, but depending on the crop, their impact can vary (Weston and Barth 1997).

CHAPTER 3. MATERIALS AND METHODS

3.1. Greenhouse Experiment

3.1.1. Location and Materials

The experiment was carried out during winter 2018 in the greenhouse in the Department of Plant, Food and Environmental Sciences, Faculty of Agriculture, Dalhousie University, Bible Hill between February and April 2018. Seeds of Malabar spinach (*B. alba*) used in this study were purchased from Richters Herbs, Goodwood, ON, Canada. A general purpose Pro-mix BX potting medium pre-mixed with 10-14% perlite, 3-7% vermiculite and 79-87% sphagnum peat moss; urea ($\text{CO}(\text{NH}_2)_2$) at 46% nitrogen (N); triple super phosphate at 46% phosphorus (P_2O_5); muriate of potash at 60% potash (K_2O); plastic seed trays and plant pots were purchased from Co-op Country Store, Truro for the study.

3.1.2. Raising Seedlings and Transplanting

Seeds of Malabar spinach were imbibed in distilled water for 24 h to facilitate better germination. Seeds were sown in plastic seed trays filled with Pro-mix BX potting medium and watered every day. Thirty-day-old healthy seedlings with 2 to 3 leaves were transplanted into 14 cm diameter plastic pots filled with Pro-mix BX potting medium up to the top leaving one inch of headspace as shown in the Figure 3.1. Greenhouse environment conditions were set at 12 h light cycle, 118*10 FC luminous intensity and 25°C in the day and 17°C at night throughout the experiment.



Figure 3.1. Thirty-day-old seedlings transplanted into plastic pots.

3.1.3. Experimental Design and Treatment Application

A completely randomized design with six replications was used to study the effect of N rate on growth and yield of Malabar spinach. Four levels of N rate (0, 40, 80 and 120 kg ha⁻¹) were randomly assigned to each experimental pot using Minitab v. 17 (Minitab Inc. 2016). Treatment levels of N, control (0 kg ha⁻¹), low (40 kg ha⁻¹), medium (80 kg ha⁻¹) and high (120 kg ha⁻¹) were applied after 15 days of transplanting i.e. at 5 leaf stage with 2 to 3 true leaves as shown in the Figure 3.2. Along with the N, recommended dose of P₂O₅ and K₂O at the rate of 60 and 40 kg ha⁻¹ respectively, were applied in the basal dose (Thambhuraj 2001).



Figure 3.2. Plants at five leaf stage with 2 to 3 true leaves.

3.1.4. Data Collection and Harvesting

Plant height from the ground level to the youngest fully expanded leaf at the apex and stem diameter at 2 cm above the ground level using digital Vernier calliper (Mitutoyo 500 digital calliper, Mitutoyo America Corporation, Illinois, USA) were measured. Leaf length from the petiole to leaf apex, leaf chlorophyll content using SPAD 502 chlorophyll meter (Spectrum Technologies Inc., Illinois, USA) and leaf anthocyanin index using ACM-200+ anthocyanin content meter (Opti-Sciences Inc., Hudson, USA) were measured from three fully expanded leaves in every experimental pot. Repeated measurements were recorded for the above responses at 45, 60 and 75 days after sowing (DAS).

Plants were harvested at 75 DAS using a sharp clean knife to remove whole plant above the soil. Total plant yield was recorded from each plant. Leaf area (LA) was measured from three fully expanded leaves per plant using Li-3100C Leaf Area meter (Li-Cor Inc., Lincoln Nebraska, USA). Specific leaf area (SLA) was calculated from one sided

area of a fresh leaf (cm^2) divided by its oven dry mass (g) dried at 80°C for 48 h. Leaf dry matter content (LDMC) was calculated from oven dry mass (mg) of a leaf divided by its water-saturated (rehydration for at least 6 h) fresh mass (g) as described by Cornelissen et al. (2003).

3.2. Field Experiment

3.2.1. Site Description and Experimental Design

The field experiment was conducted during summer 2018 between June-September at Dalhousie University's Faculty of Agriculture experimental site located at Bible Hill, Nova Scotia, Canada ($45^\circ 23' \text{N}$, $63^\circ 14' \text{W}$). The soil at the site is a Pugwash sandy loam classified as OrthicHumo-Ferric Podzol in the Canadian soil classification (Webb et al. 1991). Prior to the field trial, the site was grown with annual ryegrass (*Lolium multiflorum*) for three years. Land was prepared by disking and formation of 15 cm high raised beds with 1 m of width and 9 m length.

The experiment was laid out as split plot design (3×4) in three blocks. Each block was divided into three whole plots and randomly assigned to three planting dates (early, mid and late). Further, each whole plot was divided into four sub-plots of 1 m^2 experimental area and N rates (0, 40, 80 and 120 kg ha^{-1}) were randomly assigned. Both whole plots and sub plots were separated by 1 m^2 of bare soil strips (check) to prevent lateral flow of fertilizer treatment.

3.2.2. Soil Sampling and Nutrient Analysis

A composite soil sample was collected in an area of $9 \times 19 \text{ m}^2$, at a depth of 0-15 cm from the ground level before planting. The concentration of plant available mineral

nutrients in the soil was analyzed by The Nova Scotia Department of Agriculture Laboratory Services, Harlow Institute, Truro. Inductively coupled plasma optical emission spectrometer (ICP-OES-725, Agilent, California, USA) was used following extraction using Mehlich III method (AgroEcoLab 2016). Soil water pH and lime requirement was determined using Adams-Evans buffer by pH analyser (LabFit, Perth, Australia). Additionally, soil samples collected from the whole plots were analyzed for soil inorganic-N i.e. ammonium (NH_4^+) and nitrate (NO_3^-) using Technicon Auto-Analyser (Technicon Industrial Systems Corp., Tarrytown, NY) after extracting in 2M potassium chloride solution. Also, total-N was analysed through combustion using an elemental analyser (VarioMax CN, Elementar Americas Inc., Ronkonkoma, NY).

3.2.3. Transplanting of Seedlings, Treatment Application and After Care

Thirty-day-old healthy seedlings were raised in three different time intervals in the greenhouse as described previously. Prior to field transplanting, 23-day-old seedlings were hardened-off for one week under atmospheric day and night temperature conditions as shown in the Figure 3.3. Initially, seedlings were exposed to direct sunlight for 8 h and gradually extended to night cold temperatures. However, seedlings were moved to greenhouse when night temperatures were freezing. Water was supplied limitedly but did not allow seedlings to wilt.



Figure 3.3. Twenty-three-day-old seedlings of Malabar spinach kept for hardening.

Malabar spinach seedlings were transplanted into the field on three planting dates i.e. early (15 June – 3 August), mid (6 July – 20 August) and late (4 August – 18 September) as in the Figure 3.4. Plants were spaced at 0.3×0.3 m, accommodated 16 seedlings in an experimental plot of 1 m² area. After 15 days of field transplanting urea was applied into the soil at the rate of 0, 40, 80 and 120 kg ha⁻¹ in a single basal dose. Black polythene mulch was used to cover bare soil strips to suppress weeds growth as shown in the Figure 3.4. Plants were regularly watered using micro jets and weeds were removed using hand hoes. Observations were recorded with respect to any incidence of pests and diseases.



Figure 3.4. Malabar spinach transplanted in different planting date.

3.2.4. Data Collection and Harvesting

Plant height, leaf length, leaf chlorophyll, leaf anthocyanin, and stem diameter were measured as described in greenhouse experiment repeatedly at 45, 60 and 75 DAS. Number of branches, LDMC and total plant yield were measured at the final harvest i.e. at 75 DAS, as mentioned in the greenhouse experiment. All plant growth responses were measured from four plants selected in the middle of each experimental unit by making sure to eliminate edge effects. All leaf measurements were recorded randomly from 20 leaves from four plants. Weather data during crop growth period for Truro location was retrieved from Government of Canada website (Environmental Canada 2018). Weather data included mean day and night temperatures, number of rainfall days and total rainfall. Mean day length was retrieved from time and date website (Time and Date 2018).

3.3. Plant Tissue Analysis

3.3.1. Plant Sampling, Processing and Storage

Plant tissue samples collected from the field experiment were analyzed for total phenolic content (TPC), total carotenoid content (TCC) and nitrate content to study the treatment effects on nutritional value of Malabar spinach. Harvested plants were transferred to Dalhousie Plant, Food and Environmental Science laboratory for sampling, processing and storage. Plants were washed thoroughly with deionized water and drained. Fresh leaves and tender stems were chopped into small pieces of approximately 2 to 3 cm length. A homogeneous sample was obtained from bulking four plants. Samples were flash frozen using liquid nitrogen for 60 s and transferred immediately into sealed plastic bags before storage at -20°C. About 150 g portion of samples were freeze dried using Dura-Stop MP Freeze Tray Dryer (TD5C0C18A0, FTS systems, Stone Ridge, NY, USA) at -40°C for 36 h followed by 10°C for 12 h. Freeze dried samples were powdered using a coffee grinder and stored at -20°C in sterilized containers for tissue analysis.

3.3.2. Total Phenolic Assay

Total phenolic content was measured according to the Folin–Ciocalteu method (Singleton and Rossi 1965) and modified by Rupasinghe et al. (2010). Twenty-mL of 80% methanol was used to extract the phenolics from approximately 0.6 g of freeze-dried tissue samples in 50 mL capacity Falcon™ plastic vials. The mixtures were vortexed for 30 s followed by sonication with a VWR Scientific 750D 7.5 Gallon Aquasonic Ultrasonic Cleaner (750D, VWR, Radnor, PA, USA) for 15 min in two cycles with a 10 min cooling period between each sonication. The crude extract was vortexed for 30 s and centrifuged at 5000 rpm for 10 min using Sorvall ST 16

centrifuge (Thermo Fisher Scientific Inc, Waltham, MA, USA). An aliquot of the supernatant was collected into new vials. Gallic acid was used for the generation of a standard curve. Gallic acid stock solution was prepared fresh under dark conditions using amber colored glassware. Using the extraction solvent (80% methanol) stock solution was diluted to 10, 20, 40, 80, 100, 150 and 250 mg L⁻¹ concentrations. Twenty- μ L of extract samples, blank (80% methanol) and gallic acid standard were mixed with 100 μ L of 0.2 N Folin-Ciocalteu's phenol reagent in a clear CorningTM CostarTM 96-Well EIA/RIA Plate (COSTAR 9017, Fisher Scientific, Ottawa, ON, Canada) and gently mixed. After a 5 min incubation under room temperature, 80 μ L of 7.5% (w/v) sodium carbonate solution was added. The mixture was incubated for 2 h under room temperature and dark conditions before absorption was measured at 760 nm using a UV/VIS spectrophotometer (Tecan Infinite[®] M200 PRO, Morrisville, NC, USA). Results were expressed as mg of gallic acid equivalent (GAE) g⁻¹ DW.

3.3.3. Total Carotenoid Assay

The total carotenoid content was measured according to a method modified from Rivera and Canela (2012). Fifteen-mL of 6:4 methanol:ethyl acetate solution was added to 50 mg of freeze-dried samples and incubated at 60°C for 20 min with continuous shaking in an incubator shaker (New BrunswickTM Innova[®] 42, Eppendorf, Mississauga, ON, Canada). The samples were brought down to room temperature and the liquid phase was removed into a new glass vial. Ten-mL of the liquid phase and 10 mL of hexane-diethyl ether (9:1 v/v) were mixed and shaken after which 20 mL of saturated sodium chloride solution was added. The mixture was shaken and allowed to separate into aqueous and organic layers. The organic phase was removed, and 1 mL of extract

evaporated with N₂ gas. The dried extract was dissolved in 1 mL of 99% methanol. An aliquot of 200 µL was put into a clear Corning™ Costar™ 96-Well EIA/RIA Plate. Absorbance was measured by a UV/VIS spectrophotometer at 470 nm. The total carotenoid content was calculated according to a formula based on Gross (1991) given below and results were expressed in mg g⁻¹ DW.

$$C = \frac{Abs \times 10^4 \times V}{A_{1cm}^{1\%} \times W}$$

Where Abs = absorbance measured at 470 nm; 10⁴ = conversion factor to obtain the concentration in units of µg g⁻¹; V = volume of extract (mL); W = sample weight (g); A_{1cm}^{1%} = absorption coefficient. Lutein is the major carotenoid in Malabar spinach (Raju et al. 2007) therefore, absorption coefficient of 2550 was used.

3.3.4. Nitrate Assay

Nitrate in plant tissue samples were measured by nitration of salicylic acid by Cataldo et al. (1975) and Zhao and Wang (2017). About 100 mg of freeze-dried sample was extracted in 5 mL deionized water by boiling at 100°C for 20 min. Samples were centrifuged at 5000 rpm for 10 min and 0.1 mL of supernatant was collected into a new 15 mL Falcon™ plastic vials. About 0.4 mL of 5% (w/v) salicylic acid – sulphuric acid was added to the mixture and incubated for 20 min at room temperature. Furthermore, 9.5 mL of 8% (w/v) sodium hydroxide solution was added into each 15 mL tube and cool down the tubes to room temperature (about 20-30 min). An aliquot of 200 µL of the mixture was put into a clear Corning™ Costar™ 96-Well EIA/RIA Plate. Absorbance was measured by a UV/VIS spectrophotometer at 410 nm. Potassium nitrate was used for the generation of a standard curve using the extraction solvent (deionized water) and diluted to 10, 20, 30, 40, 60, 80, 100 and 120 mg L⁻¹

concentrations. Total NO_3^- content was calculated using standard curve and expressed in mg kg^{-1} DW.

3.4. Statistical Analysis

In the greenhouse experiment, a completely randomized design was used to study the effects of N rate and DAS. In the field experiment, split-plot design was used to study the effects of planting date and N rate which were considered as fixed and block was considered as random along with DAS. Total N, NH_4^+ -N and NO_3^- -N results from the initial soil test before the treatment applications were used as covariates. For tissue analysis, samples were measured in triplicates. Response variables that were measured repeatedly, the most appropriate covariance structure among compound symmetry (CS), unstructured (UN) and auto-regressive order (1) (AR-1) was determined using the AIC and BIC values. Analysis of variance (ANOVA) was performed for each response variable using MIXED procedure of SAS (SAS Institute Inc. 2016). Multiple means comparison was completed for significant ($P\text{-value} \leq 0.05$) or marginally significant ($P\text{-value} = 0.05$ to 0.1) effects by comparing the least squares means of the corresponding treatment combinations using the lsmeans statement of Proc MIXED with pdiff option to produce P-values for all pairwise differences. Whereas, adjust=Tukey was used with lsmeans statement for greenhouse experiment. For the response variables that were measured at once, ANOVA was completed followed by multiple means comparison. Letter groupings were generated using a 5% level of significance. For each response, the validity of model assumptions on the error terms was verified by examining the residuals as described in Montgomery (2017) and the appropriate transformations were applied on responses with violated assumptions. Weather data were expressed as averages.

CHAPTER 4. RESULTS

4.1. Greenhouse Experiment

4.1.1. Analysis of Variance Results

Analysis of variance results for the main and interaction effects of nitrogen (N) rate and days after sowing (DAS) on measured responses, shown in Table 4.1, indicated a significant N rate by DAS interaction effect on Malabar spinach plant height, anthocyanin index and marginally significant for stem diameter. Main effects of N rate and DAS were significant for leaf chlorophyll content and leaf length respectively.

Table 4.1. Analysis of variance P-values for the main and interaction effects of nitrogen (N) rate and days after sowing (DAS) on Malabar spinach plant height, leaf length, chlorophyll content, anthocyanin index and stem diameter responses.

Source of variation	Plant height	Leaf length	Stem diameter	Chlorophyll content	Anthocyanin index
N rate	0.136	0.141	0.351	0.053 ^a	0.342
DAS	<.001	<.001 ^a	<.001	0.263	0.004
N rate × DAS	0.001 ^a	0.164	0.086 ^b	0.102	0.045 ^a

^a Significant main and interaction effects with P-value ≤ 0.05 that require multiple means comparison.

^b Marginally significant effect with P-value 0.05 to 0.1 that requires multiple mean comparison.

Analysis of variance results for the response variables leaf area (LA), leaf dry matter content (LDMC), specific leaf area (SLA) and total yield measured at the final harvest were significant for the main effect N rate alone as shown in the Table 4.2.

Table 4.2. Analysis of variance P-values for the effect of nitrogen (N) rate on Malabar spinach leaf area (LA), total yield, leaf dry matter content (LDMC) and specific leaf area (SLA).

Source of variation	LA	LDMC	SLA	Total Yield
N rate	0.031 ^a	<.001 ^a	0.001 ^a	0.003 ^a

^a Significant main effect with P-value ≤ 0.05 that require multiple means comparison.

4.1.2. Plant Height, Stem Diameter, Leaf Area and Leaf Length

The plant growth determined by plant height, stem diameter and leaf area increased significantly as N rate was increased from low (40 kg N ha⁻¹) to high (120 kg N ha⁻¹). Plant growth was significantly high in the medium N fertilized plants followed by the high, low and the control N rates as shown in Figure 4.1 and Table 4.3. The growth trends for plant height and stem diameter were similar (Figure 4.1A-B) and continuously rose throughout the crop duration irrespective of the applied N rates. Also, the leaf elongation was significantly increased until final harvest, which was 3.6 cm, 9.0 cm and 13.3 cm at 45, 60 and 75 DAS, respectively.

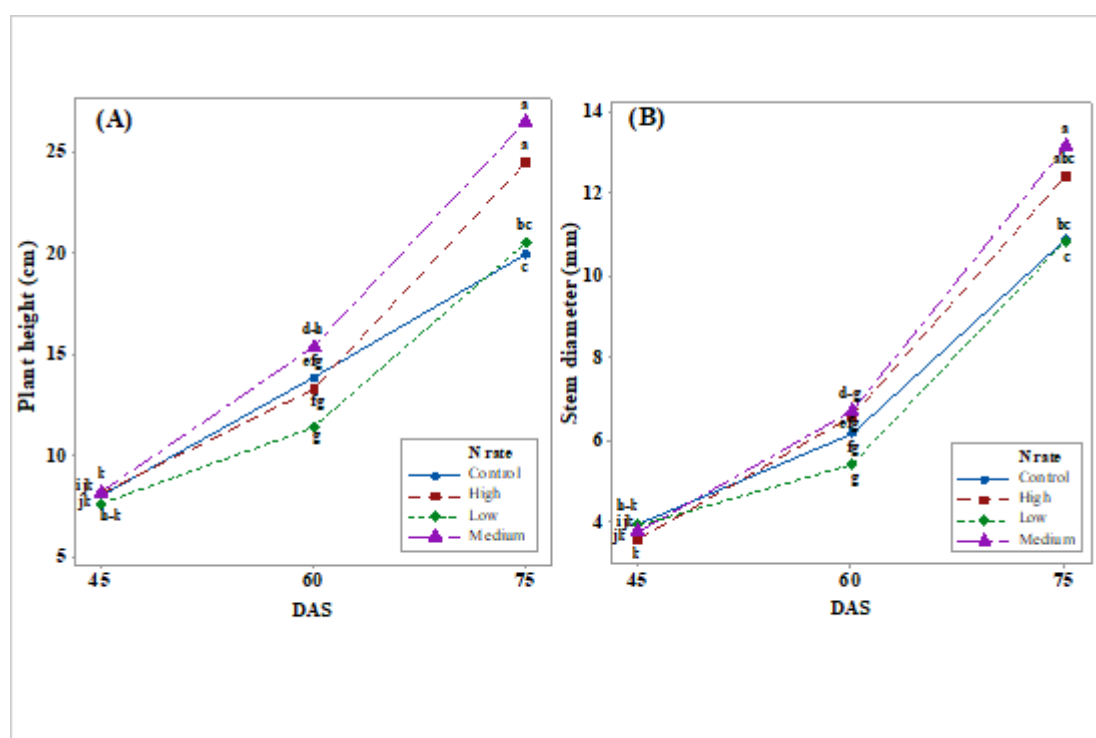


Figure 4.1. Interaction plot of nitrogen (N) rate and days after sowing (DAS) on (A) plant height and (B) stem diameter of Malabar spinach. Means sharing the same letter are not significantly different at the 5% level of significance. h-k represents hijk.

The highest plant height of 24.5 cm, stem diameter of 13.1 mm and leaf area of 101.2 cm² were achieved at the final harvest from the medium N applied plants (80 kg N ha⁻¹). There was 31.1%, 20.1% and 103.6% increase in plant height, stem diameter and

leaf area of the medium N applied plants respectively, compared to the control plants. However, there was no further increase in all the plant growth components when N rate was increased above the medium N level.

4.1.3. Leaf Chlorophyll Content and Anthocyanin Index

An increase in N rate had negative impact on leaf chlorophyll content (Table 4.3). Highest mean leaf chlorophyll of 29.9 SPAD value was recorded from plants that did not receive N fertilizer and a lowest 23.6 SPAD value was recorded from plants applied with high N rate (Table 4.3). Thus, there was a 23% reduction in leaf chlorophyll content when N application rate was increased from the control to the medium N rate.

Table 4.3. Effect of nitrogen (N) rate on mean chlorophyll content, leaf area (LA), specific leaf area (SLA), leaf dry matter content (LDMC) and total yield of Malabar spinach.

N rate (kg ha ⁻¹)	Chlorophyll	LA (cm ²)	SLA (cm ² g ⁻¹)	LDMC (mg g ⁻¹)	Total yield (g plant ⁻¹)
	content (SPAD value)				
Control	29.9 a ^z	49.7 c	127.6 b	91.5 a	55.5 c
Low	25.6 abc	66.9 bc	210.1 a	54.0 b	91.5 bc
Medium	24.3 bc	101.2 a	254.4 a	43.5 cd	176.0 a
High	23.6 c	80.8 abc	243.6 a	41.1 d	178.2 a

^z Means sharing the same letter within each column are not significantly different at the 5% level of significance.

The interaction effect of N rate and DAS on leaf anthocyanin index showed interesting drifts. The anthocyanin index of control plants remained unchanged up to the final harvest. The high and medium N applied plants recorded maximum anthocyanin index at 60 DAS and tended to decline thereafter (Figure 4.2). The low N fertilized plants showed upward trend in the accumulation of anthocyanin. However, there was no significant difference in mean anthocyanin index of the control, the low and the high N treated plants at the time of final harvest. But there was a 32.2% decrease in mean

anthocyanin index of medium N applied plants, which was 3.1 ACI (anthocyanin content index) compared to the 4.1 ACI of the low N applied plants (Figure 4.2).

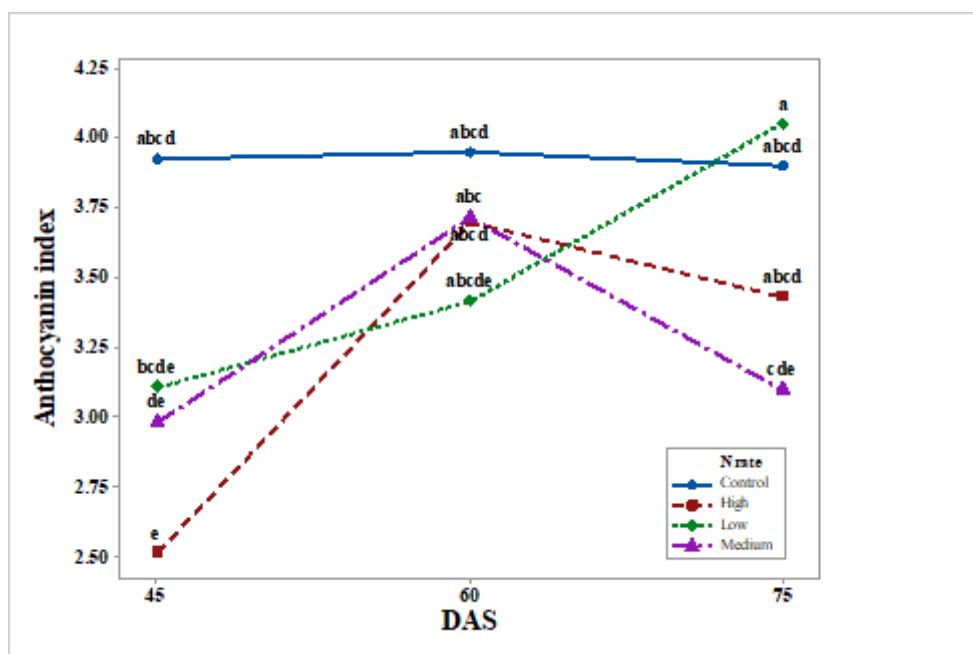


Figure 4.2. Interaction plot of nitrogen (N) rate and days after sowing (DAS) on Malabar spinach leaf anthocyanin index. Means sharing the same letter are not significantly different at the 5% level of significance.

4.1.4. Leaf Dry Matter Content and Specific Leaf Area

Nitrogen application significantly reduced LDMC in Malabar spinach as shown in Table 4.3. The highest leaf dry matter accumulated in the control plants was 91.5 mg g^{-1} and this was significantly reduced to 54 mg g^{-1} with the increase in N rate from the control to the low (Table 4.3). The lowest LDMC of 41.1 mg g^{-1} was recorded in the high N rate applied plants. However, there was no significant difference between the LDMC of the medium and the high N rate applied plants. On the contrary, there was a 64.6% increase in the SLA of Malabar spinach with the application of N fertilizer. Lowest mean SLA of $127.6 \text{ cm}^2 \text{ g}^{-1}$ was recorded from the control plants and the highest of $254.4 \text{ cm}^2 \text{ g}^{-1}$ was recorded from the high N rate applied plants. However, there were no significant differences in the SLA values among the plants applied with different N levels (Table 4.3).

4.1.5. Total Yield

The highest plant yield of 178.2 g plant⁻¹ was produced from the medium N rate applied plants which was 80 kg ha⁻¹ (Table 4.3). Application of 80 kg N ha⁻¹ increased the plant yield by 221% when compared to the control plants, which gave the lowest Malabar spinach yield of 55.5 g plant⁻¹. However, further increase in N rate from 80 kg ha⁻¹ to 120 ha⁻¹ did not change the plant yield.

4.2. Field Experiment

4.2.1. Soil Nutrient Status

The two soil macronutrients phosphorus (P₂O₅) and potash (K₂O) were higher (Table 4.4) than the recommended dose of 60 and 40 kg ha⁻¹ respectively, for Malabar spinach (Thambhuraj 2001). Hence, P₂O₅ and K₂O external fertilizers were not applied to the plants. Other minor mineral nutrients were not limiting, and the soil organic matter was in fair amount (Table 4.4). Soil pH was 6.38 and acceptable for Malabar spinach production.

Table 4.4. Soil mineral, organic matter and pH status before the trial at the experimental site.

Parameter	Analysis
Depth (cm)	0-15
P ^a (kg/ha)	573
K ^b (kg/ha)	396
Ca ^c (kg/ha)	2532
Mg ^d (kg/ha)	267
Na ^e (kg/ha)	22
S ^f (kg/ha)	26
Al ^g (ppm)	1579
B ^h (ppm)	<0.5
Cu ⁱ (ppm)	1.4
Fe ^j (ppm)	124
Mn ^k (ppm)	60
Zn ^l (ppm)	2.21
Organic Matter (%)	3.5
pH	6.38

^a Phosphorous, ^b potash, ^c calcium, ^d magnesium, ^e sodium, ^f sulfur, ^g aluminum, ^h boron, ⁱ copper, ^j iron, ^k manganese, ^l zinc.

4.2.2. Environmental Conditions

Table 4.5 showed the climatic conditions at the experimental site located in Bible Hill, Truro, NS during the field experiment. The highest temperatures were recorded between July and the first half of August. Mean day length in the month of June-July was at the highest and began to fall thereafter. Rainfall days were evenly distributed throughout the crop duration. However, the amount of rainfall received in late June was highest i.e. 122.2 mm followed by the least of 15.8 mm in the first half of July.

Table 4.5. Air temperatures, day length, rainfall days and total amount of rainfall during the field trial.

Crop duration (Biweekly)	Air temperatures (°C)			Mean day length (h d ⁻¹)	Rainfall days	Total rainfall (mm)
	Maximum	Minimum	Mean			
	June 15 - June 30	28.4	4.6	14.6	15.3	5
July 1 - July 15	32.9	7.6	19.0	15.2	2	15.8
July 16 - July 31	30.5	11.7	21.8	15.1	5	31.6
Aug 1 - Aug 15	32.7	9.0	21.8	14.2	5	31.1
Aug 16 - Aug 31	29.2	7.7	18.4	13.4	5	33.7
Sept 1 - Sept 18	27.7	1.6	16.0	12.5	3	46.1

4.2.3. Analysis of Variance Results

Analysis of variance results for the main and interaction effects of planting date, N rate and DAS on measured responses with the covariates are shown in the Table 4.6. Plant height and leaf length of Malabar spinach in the field were significantly influenced by the interactions of planting date by N rate and planting date by DAS. Whereas, stem diameter was significantly affected by the interaction effect of planting date by DAS. Leaf chlorophyll content was significantly affected by the interactions of planting date by DAS and N rate by DAS. Three-way interaction effect of planting date, N rate and DAS was significant on leaf anthocyanin index (Table 4.6). Soil covariates used in this study did not show any significance on measured responses except for anthocyanin index where, total soil-N was significant.

Table 4.6. Analysis of variance P-values for the main and interaction effects of planting date (PD), nitrogen (N) rate and days after sowing (DAS) with total soil-N, nitrate-N and ammonium-N covariates on Malabar spinach plant height, leaf length, stem diameter, chlorophyll content and anthocyanin index responses.

Source of variation	Plant height	Leaf length	Stem diameter	Chlorophyll content	Anthocyanin index
PD	0.156	0.223	0.091	0.277	0.088
N rate	0.541	0.323	0.105	0.001	<.001
PD × N rate	0.041 ^a	0.031 ^a	0.288	0.469	<.001
DAS	<.001	<.001	<.001	<.001	<.001
PD × DAS	<.001 ^a	<.001 ^a	<.001 ^a	<.001 ^a	<.001
N rate × DAS	0.443	0.621	0.605	0.052 ^a	<.001
PD × N rate × DAS	0.184	0.336	0.718	0.395	0.001 ^a
Total soil N	0.316	0.975	0.747	0.811	0.023 ^b
Soil nitrate - N	0.193	0.991	0.873	0.637	0.061
Soil ammonium - N	0.891	0.695	0.203	0.733	0.212

^a Significant main and interaction effects with P-value ≤ 0.05 that require multiple means comparison.

^b Significant covariate with P-value ≤ 0.05 .

Interaction effect of planting date by N rate was significant for number of branches and LDMC whereas, marginally significant for total yield measured during the final harvest (Table 4.7).

Table 4.7. Analysis of variance P-values for the main and interaction effects of planting date (PD) and nitrogen (N) rate with total soil-N, nitrate-N and ammonium-N covariates on Malabar spinach number of branches, leaf dry matter content (LDMC) and total yield responses.

Source of variation	Number of branches	LDMC	Total yield
PD	0.321	0.383	0.137
N rate	0.634	0.638	0.155
PD × N rate	0.031 ^a	0.011 ^a	0.085 ^b
Total soil N	0.633	0.289	0.966
Soil nitrate - N	0.111	0.446	0.861
Soil ammonium - N	0.787	0.926	0.201

^a Significant main and interaction effects with P-value ≤ 0.05 that require multiple means comparison.

^b Marginally significant effect with P-value 0.05 to 0.1 that requires multiple mean comparison.

4.2.4. Plant Growth

Malabar spinach plant height, leaf length and number of branches at an early planting were significantly increased by the application of low N rate of 40 kg ha⁻¹ (Figure 4.3) compared to the control. There was a 16.5% increase in plant height, 18.1% increase in leaf length and 66.6% increase in the number of branches of low N fertilized plants compared to the control plants. However, further increase in the application rate of N did not improve plant growth in early planting (Figure 4.3). Therefore, the highest plant height recorded was 14.8 cm, leaf length was 9.8 cm and number of branches was 10 at the final harvesting of early plants.

Application of N rates did not influence the growth of Malabar spinach in the mid and late plantings. Plant height, leaf length and the number of branches achieved in the control plants were not significantly different from the N applied plants (Figure 4.3). Also, the application of different N rates ranging from low to high did not enhance plant growth. Additionally, there were no significant differences in the leaf length and number of branches of the control plants from mid to late planting (Figure 4.3B-C).

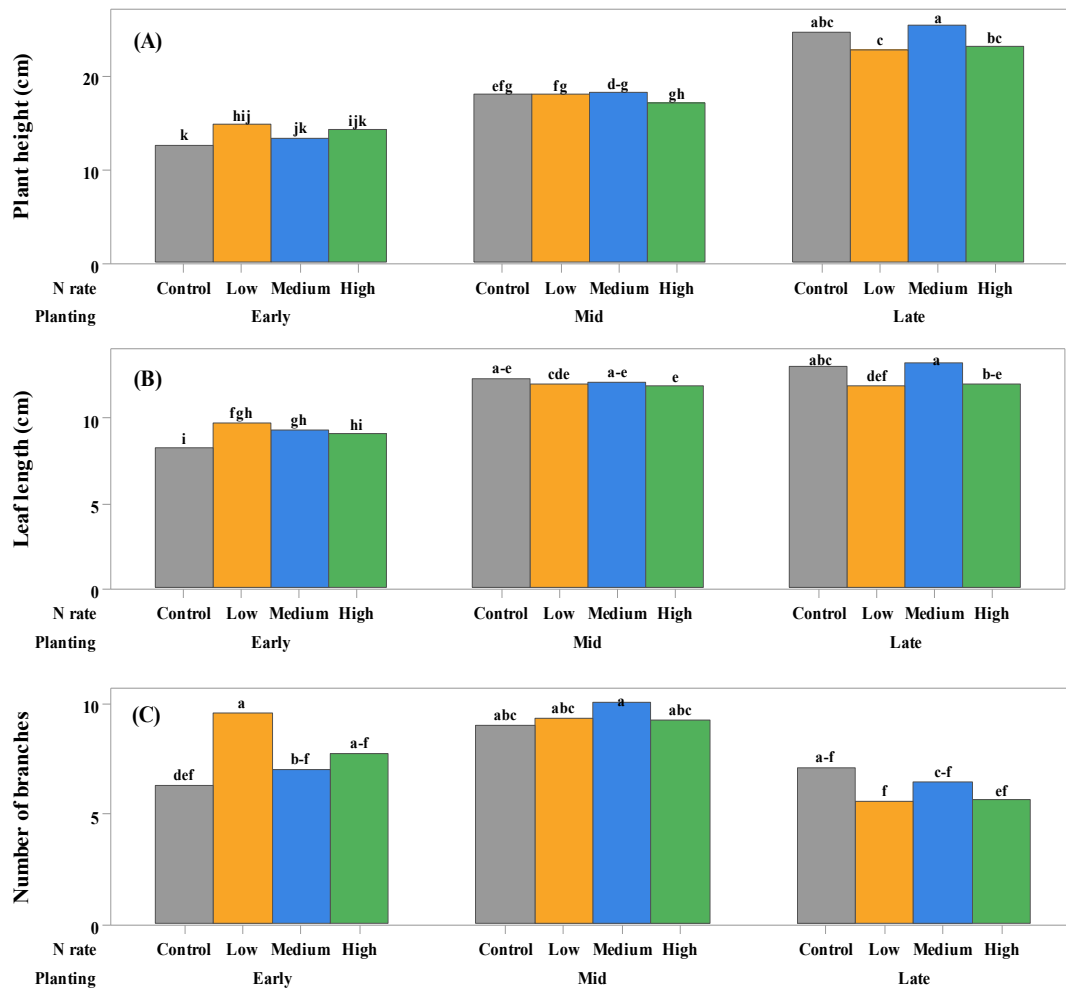


Figure 4.3. Interaction plots of planting date and nitrogen (N) rate on (A) plant height, (B) leaf length and (C) number of branches of Malabar spinach. Means sharing the same letter are not significantly different at the 5% level of significance. d-g represents defg.

The highest leaf length achieved was 13 cm and the number of branches was 9. However, plant height was an exception. Mean plant height of the control plants in late planting was increased by 36.4% compared to the control plants in mid planting (Figure 4.3A). Overall, plant growth in terms of plant height and leaf length was significantly high in mid and late plantings compared to early planting (Figure 4.3A-B).

For the interaction of planting date by DAS, plant height and stem diameter increased continuously throughout the growing period irrespective of the planting date. At the

time of final harvest, mean plant height ranged from 26.3 to 32.9 cm and stem diameter ranged from 14.3 to 18.5 mm (Figure 4.4A-B). The highest plant height (32.9 cm) and stem diameter (18.5 mm) were recorded in mid plantings but, this was not significantly different from late planting. Whereas, the lowest plant height (26.3 cm) and stem diameter (14.3 mm) recorded in early planting were significantly reduced by 25.09% and 29.03% in growth respectively compared to mid and late plantings (Figure 4.4A-B).

On the other hand, leaf lengths of early and mid-season showed continuous increase in elongation whereas in the late planting, leaf growth tended to decline after 60 days of sowing (Figure 4.4 B). Leaf length was reduced by 32.8% in the final harvested plants of the late planting compared to that of the early and mid-season. Overall, leaf length at the final harvest ranged from 13.1 to 17.4 cm, the highest leaf length was recorded for the late planting, which was not significantly different from that of the early planting.

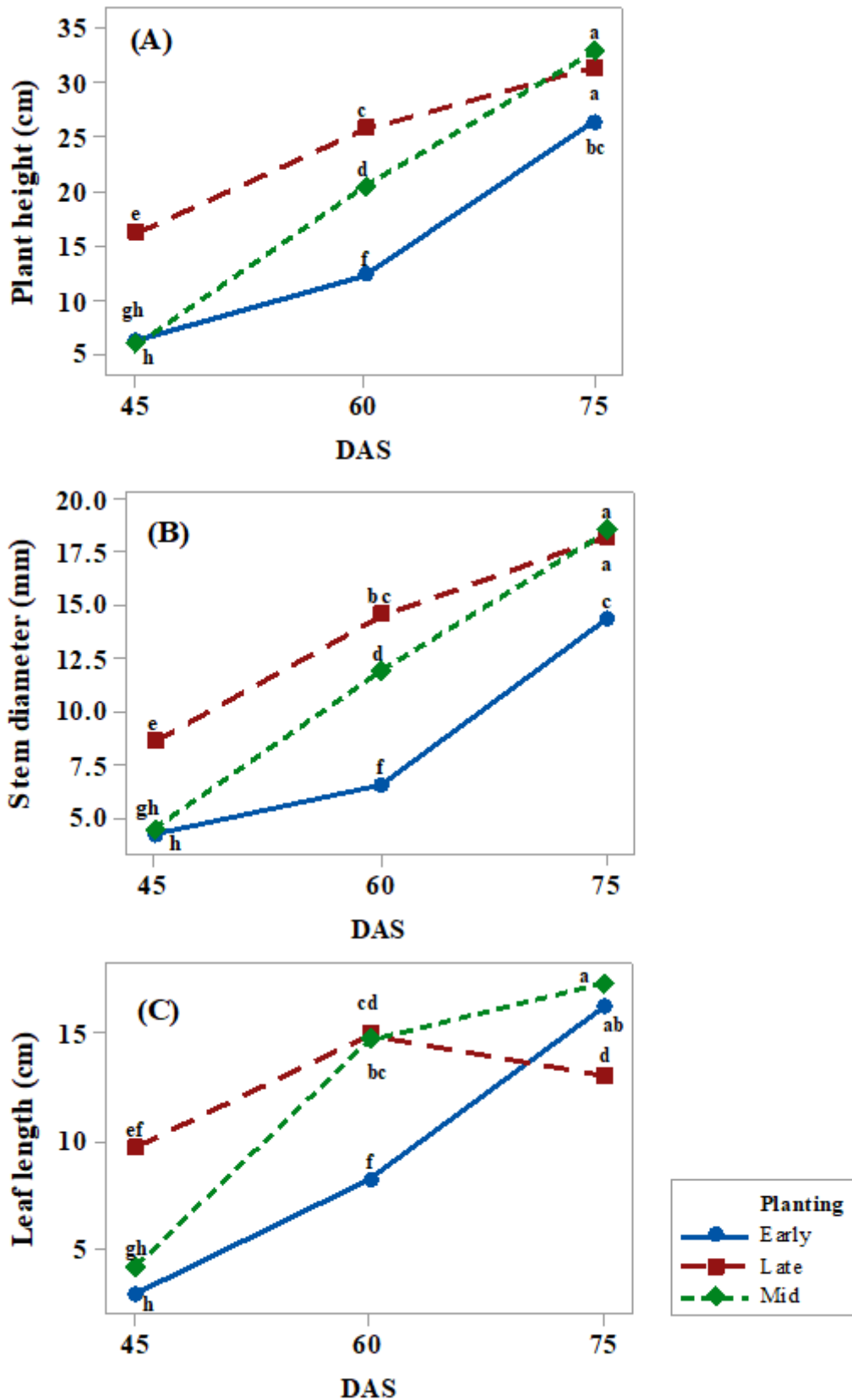


Figure 4.4. Interaction plots of planting date and days after sowing (DAS) on (A) plant height, (B) stem diameter and (C) leaf length of Malabar spinach. Means sharing the same letter are not significantly different at the 5% level of significance.

4.2.5. Leaf Chlorophyll Content

In every planting, synthesis and accumulation of leaf chlorophyll was constantly increased until the crop harvested (Figure 4.5A). Leaf chlorophyll content was highest upon late planting with 47.1 SPAD and it was significantly high compared to early and mid-season with the mean chlorophyll content of 36.9 SPAD.

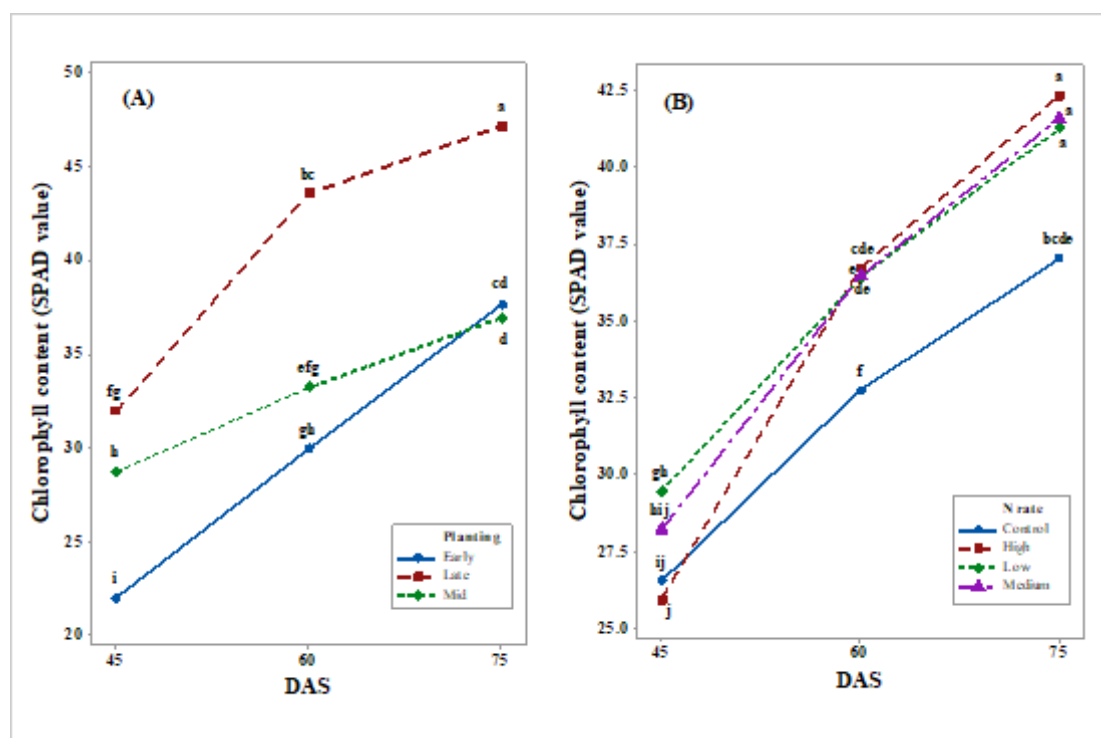


Figure 4.5. Interaction plot of (A) planting date and days after sowing (DAS), (B) nitrogen (N) rate and DAS on leaf chlorophyll content of Malabar spinach. Means sharing the same letter are not significantly different at the 5% level of significance.

In the field experiment, N application substantially increased leaf chlorophyll content in Malabar spinach (Figure 4.5B). Overall, leaf chlorophyll significantly increased to 42.3 SPAD with the application of N fertilizer and this increment was 14.3% higher compared to the control plants (0 kg N ha^{-1}). However, there was no significant differences in leaf chlorophyll content among the plants applied with the different N rates (Figure 4.5B).

4.2.6. Leaf Anthocyanin Index

Three-way interaction of planting date, N rate and DAS was significant for leaf anthocyanin index (Table 4.6). Interestingly, the covariate total soil-N used in this study showed significance only for the leaf anthocyanin index (Table 4.6). Nitrogen application did not show any effect on anthocyanin synthesis during early and mid-plantings. The maximum leaf anthocyanin accumulated in the early planting was 6.2 ACI, and that for the mid planting was 5.3 ACI at final harvesting (Figure 4.6). However, in late planting, N application showed significant effect on anthocyanin accumulation. Highest anthocyanin of 11.2 ACI was recorded under low N application, followed by medium and then the high N rates with mean leaf ACI of 9.5 and 8.3, respectively. Additionally, the plants from the control treatment showed decline in leaf anthocyanin index to 5.8 after 60 days of sowing.

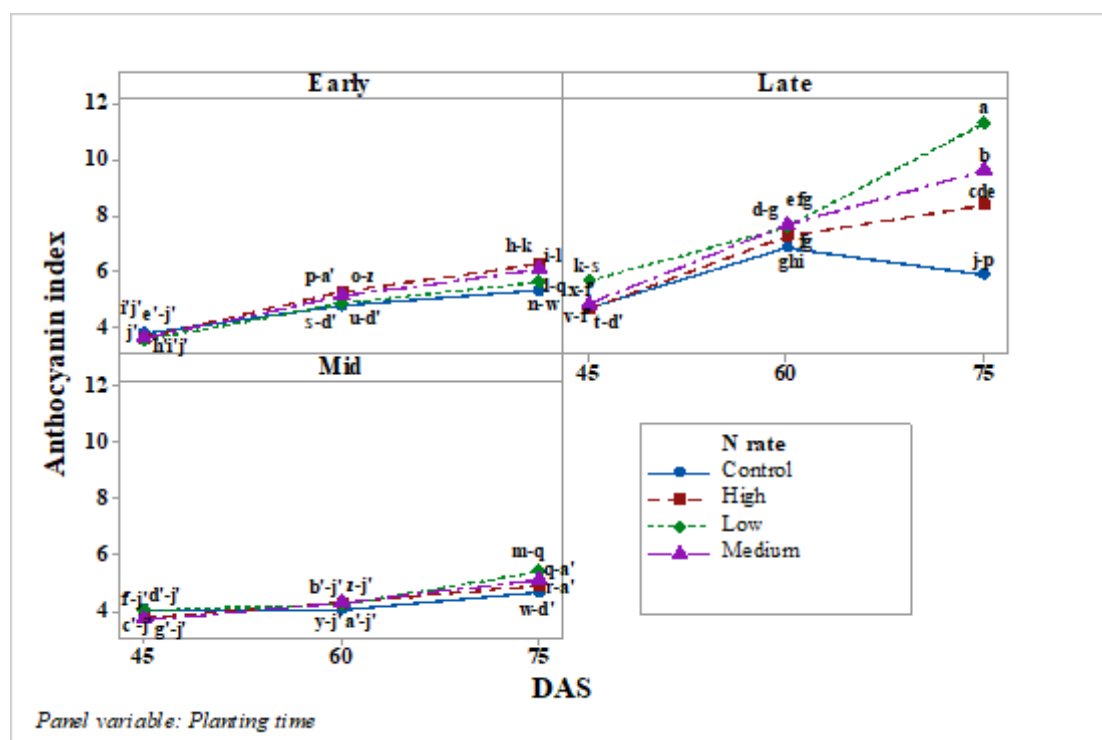


Figure 4.6. Interaction plot of planting date, nitrogen (N) rate and days after sowing (DAS) on leaf anthocyanin index of Malabar spinach. Means sharing the same letter are not significantly different at the 5% level of significance. s-d' represents stuvwxyzab'c'd'.

4.2.7. Leaf Dry Matter Content and Total Yield

Planting date did not show any effect on the accumulation of LDMC whereas, N rate showed little effect (Table 4.8). Although there was not statistically significant difference across the planting date and N rate, there was moderate increase in LDMC of the control plants and an overall reduction in LDMC when N rate was increased. Leaf dry matter accumulated across the planting date ranged from 38.3 to 63.1 mg g⁻¹ (Table 4.8). The lowest LDMC was from the mid-season control treatment and the highest was from late planted control treatment.

Table 4.8. Interaction effect of planting date and nitrogen (N) rate on mean leaf dry matter content (LDMC) and total yield of Malabar spinach

Planting date	N rate (kg ha ⁻¹)	LDMC (mg g ⁻¹)	Total yield (g plant ⁻¹)
Early	Control	52.9 c-i ^z	111.3 g
	Low	46.1 g-j	210.4 def
	Medium	49.4 d-j	185.2 ef
	High	48.1 f-j	181.6 f
Mid	Control	38.3 j	472.3 ab
	Low	49.0 e-i	436.3 abc
	Medium	45.7 hi	512.1 a
	High	43.2 ij	435.9 abc
Late	Control	63.1 a	341.7 a-d
	Low	60.3 a-g	277.4 c-f
	Medium	56.2 b-i	381.8 ab
	High	58.2 a-h	311.2 b-f

^z Means sharing the same letter within each column are not significantly different at the 5% level of significance. c-i represents cdefghi.

Total yield of Malabar spinach from the early planting ranged from 111.3 to 210.5 g plant⁻¹. The lowest yield was from control treatment and the highest yield was from low N applied treatment (Table 4.8). Irrespective of the N levels applied, yield from early planting was significantly lower than the mid and the late plantings. The plant yield for the mid and late plantings did not respond to the applied N. The highest yield of 512.1 g plant⁻¹ from the mid planting was due to medium N application, but this was not

significantly different from the control treatment (Table 4.8). Similarly, the highest yield produced in the late planting was 381.8 g plant⁻¹ from medium N rate, which was not significantly different from the control treatment.

4.3. Tissue Analysis

Analysis of variance P-value showed significant interaction effect of planting date by N rate on total phenolic content and total carotenoid content. The main effect of N rate was marginally significant on plant nitrate content (Table 4.9). The covariates total soil-N, nitrate-N and ammonium-N used in the study did not also show any significant difference.

Table 4.9. Analysis of variance P-values for the main and interaction effects of planting date (PD) and nitrogen (N) rate with covariates total soil nitrogen (N), nitrate-N and ammonium-N on total phenolic content (TPC), total carotenoid content (TCC) and nitrate content of Malabar spinach.

Source of variation	TPC	TCC	Nitrate content
PD	0.201	0.388	0.632
N rate	0.049	0.03	0.068 ^b
PD × N rate	0.041 ^a	0.022 ^a	0.229
Total soil N	0.872	0.886	0.57
Soil nitrate - N	0.552	0.334	0.725
Soil ammonium - N	0.709	0.551	0.664

^a Significant interaction effect with P-value ≤ 0.05 that require multiple means comparison.

^b Marginally significant main effect with P-value 0.05 to 0.1 that requires multiple mean comparison.

4.3.1. Total Phenolic Content and Total Carotenoid Content

The total phenolic content was significantly high in the early planting date compared to the late planting date (Table 4.10). There was no effect of applied N rates on the total phenolic content in the early planting, but there was a significant decrease in phenolic content of the mid and the late plantings when the N rate was increased from control to

high. Also, Table 4.10 showed declining of phenolic content toward mid to late plantings. Total phenolic content across the planting date was varied from 4.88 to 7.65 mg GAE g⁻¹ dry weight (DW). The highest phenolic content was recorded in the control treatment of early planting whereas, the lowest was recorded in the high N rate applied plants of the late planting (Table 4.10).

Table 4.10. Interaction effect of planting date and nitrogen (N) rate on mean total phenolic content (TPC) and total carotenoid content (TCC) of Malabar spinach.

Planting date	N rate (kg ha ⁻¹)	Moisture (%)	TPC (mg GAE g ⁻¹ DW)	TCC (mg g ⁻¹ DW)
Early	Control	94.8	7.65 a ^z	1.13 j
	Low	95.4	7.19 ab	1.44 f-i
	Medium	95.1	7.12 abc	1.30 hij
	High	95.2	7.52 ab	1.28 ij
Mid	Control	96.2	6.73 bc	1.54 zd-i
	Low	95.2	6.33 c-f	1.52 ze-i
	Medium	95.5	7.07 ab	1.80 abz
	High	95.7	5.90 d-g	1.54 zc-i
Late	Control	93.6	5.51 efg	1.42 g-j
	Low	93.9	5.41 fgh	1.45 zf-j
	Medium	94.3	5.05 gh	1.55 zb-j
	High	94.1	4.88 h	1.76 ac-f

^z Means sharing the same letter within each column are not significantly different at the 5% level of significance. c-f represents cdef; DW, dry weight.

For the total carotenoids, planting date had no effect, but N rate had little effect. Total carotenoid content varied from 1.13 to 1.80 mg g⁻¹ DW across the planting date and N rates (Table 4.10). The highest content of carotenoid of 1.80 mg g⁻¹ DW was recorded from the mid planting date with medium N fertilized plants, which was 59% higher than the control plants in the early planting date. Overall, a considerable increase in total carotenoid content was noticed when N rate was increased, and this increment was consistent with respect to each date of planting.

4.3.2. Nitrate Content

Plant tissue nitrate content was highest in the medium N rate applied plants and lowest in the low N rate applied plants (Table 4.11). The highest nitrate content of 254.4 mg kg⁻¹ DW in the medium N rate applied plants was not significantly different from the high N rate applied plants. Also, the lowest nitrate content of 182.2 mg kg⁻¹ DW recorded from the low N rate applied plants was not significantly different from the control and the high N rate applied plants (Table 4.11).

Table 4.11. Main effect of nitrogen (N) rate on mean nitrate content of Malabar spinach.

N rate (kg ha ⁻¹)	Moisture (%)	Nitrate content (mg kg ⁻¹ DW)
Control	94.9	187.8 bc ^z
Low	94.8	182.2 c
Medium	95.0	254.4 a
High	95.0	236.7 abc

^zMeans sharing the same letter within each column are not significantly different at the 5% level of significance. DW, dry weight.

CHAPTER 5. DISCUSSIONS

5.1. Greenhouse Experiment

Nitrogen (N) is a major plant nutrient, which is involved in various plant metabolic functions. In a crop growing system, N fertilization promotes vegetative growth of plants through increased production of leaves and stems (Opiyo 2004). Our research results agreed with the above statement because the greatest increase in Malabar spinach vegetative components such as plant height, stem diameter and leaf area (Figure 4.1 and Table 4.3) were as a result of the application of 80 kg N ha⁻¹ compared to the control (0 kg N ha⁻¹) plants. The increase in vegetative growth of the Malabar spinach plants under the influence of high N fertilization is based on the pattern of plants allocation mechanism (Chapin et al. 1987). Typically, a balance between carbon (C) and N is crucial for a plant to optimally perform its metabolic activity (Zheng 2009). Therefore, in the presence of high N, plants tend to produce more shoots in order to assimilate more CO₂ through photosynthesis to yield an ultimate C source i.e. carbohydrates in the form of glucose and sucrose (Gulmon and Chu 1981; Chapin et al. 1987).

However, it was also observed that further increase in N rate from 80 kg ha⁻¹ to 120 kg ha⁻¹ did not significantly increase plant growth. This result is consistent with the previous study conducted by Wang and Li (2004), where the growth and yield of cabbage (*Brassica oleracea*) and spinach (*Spinacia oleracea*) did not continuously increase with N rate. Plants utilize N only at the optimal level and therefore, an excessive N application reduces plant growth and yield (Albornoz 2016). Our study revealed that the medium N rate (80 kg ha⁻¹) was the optimal rate to obtain maximum growth and yield of Malabar spinach in the greenhouse environment (Figure 4.1 and

Table 4.3). It was found that the total plant yield of 176 g plant⁻¹ was produced from the application of 80 kg N ha⁻¹ to Malabar spinach. A similar experiment conducted by Rop et al. (2012) reported that 90 kg N ha⁻¹ was optimum for greenhouse production of Malabar spinach.

Plant leaf chlorophyll content is greatly influenced by growing medium mineral nutrients. Of all the elements, the greatest influence in the formation of chlorophyll pigment is exerted by N nutrition (Razaq et al. 2017). Nitrogen is an important constituent of chlorophyll and therefore, N addition promotes the formation of chlorophyll pigments by enhancing the amount of stromal and thylakoid proteins in the leaf tissues (Razaq et al. 2017). On the contrary, we found that high leaf chlorophyll content was recorded from the control plants (0 kg N ha⁻¹) and there was a significant decrease in chlorophyll content as N application rate was increased (Table 4.3). This result was not surprising as there are many literature reports that show a similar trend.

A greenhouse experiment by Liu et al. (2006) showed a decline in spinach leaf chlorophyll content with the application high N rate. According to Bojović and Stojanović (2005), excessive application of N reduces leaf lifespan by increasing photosynthetic activity and speeding up leaf senescence. It is also important to note that all the Malabar spinach plants in this study were supplied with the recommended rates of phosphorous (P₂O₅) and potash (K₂O). Therefore, the influence of P₂O₅ in the formation of leaf chlorophyll cannot be neglected in the control plants as cautioned by (Razaq et al. 2017).

B. alba is a green variety of Malabar spinach with a relatively good amount of anthocyanin content as reported in previous study by Oloyede et al. (2013). Generally, anthocyanins are formed in the leaves in response to various abiotic stress conditions. Thus, the N limited condition in the control and low N fertilized plants (Figure 4.2) led to synthesis and accumulation of high anthocyanin, which conforms to an observation by Feyissa et al. (2009). Also, Stewart et al. (2001) reported a significant inverse relation between N application rate and anthocyanin content, which was similarly observed in the present study.

Specific leaf area (SLA) and leaf dry matter content LDMC indicates different plant functions and are inversely related to each other; Increased SLA is associated with decreased LDMC and *vice-versa* (Cornelissen et al. 2003; Yulin et al. 2005). Specific leaf area is an important leaf trait to compare plant ecology and it is associated with plant growth and survival. Specific leaf area is positively correlated with potential relative growth rate and leaf net photosynthetic rate but correlates negatively with leaf lifespan (Shipley and Vu 2002). Also, a greater SLA indicates resource rich environment and lower values correspond to longer lifespan (Cornelissen et al. 2003). Therefore, the high SLA recorded for the N fertilized plants suggests availability of resources including mineral nutrients compared to the control plants with lower SLA. Thus, the higher SLA as a result of increased N rate also indicated an increase in photosynthetic activity.

Results for LDMC showed significantly high dry matter content in the control plants and decreased dry matter content with increased application of N rate (Table 4.3). Similarly, in a study by Sorensen (1999) stated an inverse relation between applied N

rate and leaf dry matter accumulation in vegetables such as cabbage, carrot (*Daucus sativus*), broccoli (*Brassica oleracea* var *italica*) and leek (*Allium ampeloprasum*) which are expected. This was because high N rates reduced the accumulation of sugars and dietary fibres that are key compositions of dry matter (Sorensen 1999).

5.2. Field Experiment

Plant growth, yield and quality are very much influenced by the growing environmental conditions such as light intensity, water availability, soil and air temperatures, photoperiod and availability of plant nutrients. In the interaction of planting date by N rate it was observed that the growth of Malabar spinach height, leaf length and branch numbers during early planting was significantly decreased compared to mid and late plantings (Figure 4.3). The mean atmospheric temperatures during the early planting date were relatively low; Night temperatures during seedling establishment were below 5°C (Table 4.5). The optimum day temperature for the growth of Malabar spinach is between 20°C and 35°C and night temperature is above 10°C (AVRDC 2003). Therefore, the air temperatures during the early planting date were not within the acceptable temperature for Malabar spinach growth and development.

Additionally, the absorption and translocation of mineral nutrients by plants from the soil are affected by soil and air temperatures. The soil temperature (data not presented) during early plant date was low due to the low atmospheric temperature. An experiment conducted to study temperature effect on the absorption of ammonium (NH_4^+) and nitrate (NO_3^-) demonstrated an increased uptake of ions from the growing medium when air and root temperatures increased from 8°C to 23°C. It further explained that NO_3^- uptake rate was more affected by root zone temperature than NH_4^+ uptake (Frota

and Tucker 1972). These can explain the results of our findings. However, low N rate i.e. 40 kg ha⁻¹ could be used as the optimal N rate to achieve maximum growth of Malabar spinach in early planting date i.e. spring (June) in Nova Scotia when soil temperature is low (Figure 4.3).

There were no differences in the growth of plant height, leaf length and number of branches between the control and the N applied plants of mid and late planting dates. Also, there was no significant difference in plant growth following application of the low, medium and high N rates applied to plants (Figure 4.3). From Table 4.5, it is evident that transplanted seedlings of the mid and the late planting dates were exposed to high temperatures during the peak summer months that contributed to increased plant growth. Additionally, Malabar spinach, a C₄ plant, has greater photosynthesis efficiency as temperature, day length and light intensity increase (Long 1998; Abukutsa-Onyango 2004). This may explain the high photosynthesis efficiency although N was limiting for the control plants under the conditions of the present study. On the other hand, a significant reduction in the leaf length of Malabar spinach grown in late planting was observed after 60 days of seed sowing (Figure 4.4C). This observation was in accordance with observations made by Whitehead et al. (2002) and Ribeiro et al. (2017). They reported similar findings where the vegetative growth and leaf elongation of vegetable amaranth (*Amaranthus tricolor*) was greatly reduced under decreasing day length and low mean daily air temperatures. Increased plant height, leaf length and stem diameter in transplanted seedlings of the late planting date was due to change in environmental conditions in the greenhouse during seedlings production.

As observed in both the greenhouse and the field experiments, Malabar spinach plant height and stem diameter were affected by the interaction of planting date and DAS were similar (Figures 4.1, 4.4A-B). This indicated that the growth of plant height was complimentary to the growth of stem diameter or *vice-versa*. This finding can be a valuable information for the future plant breeding in Malabar spinach.

The interaction effect of planting date by DAS on chlorophyll content showed high accumulation of 47.1 SPAD in the late planting compared to early and mid-season plantings (Figure 4.5A). This result can be explained based on the findings of Ali et al. (2009) where, the accumulation of chlorophyll pigments is significantly greater under 12 h photoperiod than under the range between 6 to 12 h and 18 to 24 h photoperiodic levels in red and green amaranths, spinach, Swiss chard (*Beta vulgaris* L. ssp. *cicla*) and Red beet (*Beta vulgaris* L. ssp. *vulgaris*). Therefore, increased accumulation of chlorophyll in late planting was partly as a result of reduce in day length from 15.14 to 12.59 h day⁻¹ as shown in the Table 4.5. Furthermore, chlorophyll content for the interaction of N rate and DAS (Figure 4.5B) was different from the greenhouse experiment (Table 4.3) due to various uncontrolled conditions on the field.

The inconsistencies in anthocyanin content cannot be explained. However, in a study conducted by Gazula et al. (2007) about the influence of planting date on anthocyanin levels in nine lettuce (*Lactuca sativa*) cultivars, they reported an increase in anthocyanin content during late summer (August-September) compared to early summer (June-July). It is also important to note that anthocyanin synthesis and accumulation in plants are sensitive to uncontrolled environmental conditions. Interaction between plant genotype and environmental factors such as light,

temperature and nutrient deficiency plays an important role in the synthesis and accumulation of anthocyanin (Steyn et al. 2002; Marin et al. 2015).

As explained for the greenhouse experiment, an inverse relationship exists between the field N application rate and LDMC, but planting date did not influence Malabar spinach dry matter accumulation (Table 4.8). In any leafy vegetables, plant vegetative growth translates into potential leaf yield (Opiyo 2004). In our study also increased growth of plant height, stem diameter, leaf length and number of branches from the corresponded treatments contributed for increase in total yield. Since there was no significant difference between the plant yield of control and N applied plants in mid and late plantings, reducing N thus appears to improve growers' profits without affecting plant growth and yield.

Plant tissue samples obtained from the field experiments were analysed for TPC, TCC and nitrate content to know the effects of planting date and N application rate. Results from our study showed variations in the phenolic concentration accumulated in leaf tissues with respect to different planting dates (Table 4.10). The variations in the phenolic compounds in tomato (*Solanum lycopersicum*) leaves and lettuce were previously attributed to seasonal weather changes (Benard et al. 2009; Marin et al. 2015). Furthermore, total phenolic compounds accumulated in the late planting was significantly low compared to the early planting date (Table 4.10). Ali et al. (2009) reported accumulation of total phenolic compounds from plants grown under 12 h photoperiod than plants compared to under 6, 18 and 24 h photoperiods. Our results appear to agree with these findings as there was a reduction in day length from 15 h to 12 h during the late planting date (Table 4.5). Additionally, total phenolic compounds

decreased with an increase in the application of N rate in our study (Table 4.10). This was not uncommon because at an increase in N application rate, plants metabolism shifts towards synthesis of N-containing protein compounds from carbon containing sugar compounds (Albornoz 2016). Overall, the phenolic concentration recorded from our experiment was less compared to the concentrations of Malabar spinach grown in tropical climatic conditions. For instance, Maisuthisakul et al. (2007) reported 15.5 mg GAE g⁻¹ DW from the buds of Malabar spinach whereas Oloyede et al. (2013) reported 28.17 mg g⁻¹ DW.

Previous studies have reported an increase carotenoid synthesis with the application of N fertilizer (Sorensen 1999). Our results confirmed this assertion. Total carotenoid content found that was found in our study was in good amount compared to spinach (1.96 mg g⁻¹ DW), amaranth (*Amaranthus caudatus*) (1.18 mg g⁻¹ DW) and curry leaf (*Murraya koenigii*) (1.99 mg g⁻¹ DW) as reported by Gunathilake and Ranaweera (2016).

Despite of N treatments applied, the amount of nitrate found in the plant tissues of Malabar spinach from our experiment was very low compared to any other leafy vegetables from the literature (Table 4.11). Beetroot, Celery (*Apium graveolens*), lettuce, spinach and Turnips (*Brassica rapa* subsp. *rapa*) are some examples of the vegetables that are known for high nitrate accumulation, which is greater than 2500 mg kg⁻¹ of fresh weight (Blom-Zandstra 1989).

CHAPTER 6. CONCLUSION AND RECOMMENDATIONS

6.1. Overview of Problem and Research Objectives

Increasing immigrant population in Canada is creating huge demand for ethnic vegetables. Since most of the ethnic vegetables are of tropical origin, there are less information available for their commercial production in Canada despite the increasingly high market demands in almost all the provinces. Research information on crop management practices such as planting date, fertilization rate, crop growth, maturity and harvesting are needed to successfully cultivate ethnic vegetables in Canada. Therefore, this research was aimed to evaluate the production potential of tropical ethnic leafy vegetable Malabar spinach under Canadian maritime climatic conditions. Also, the effect of climatic conditions of Canada on nutritional value of Malabar spinach. A greenhouse experiment was conducted to study the effect of nitrogen (N) rate on growth and yield of Malabar spinach. Another field study was conducted to know the growth, yield and chemical compositions of Malabar spinach under the influence of N rate and planting date.

6.2. Conclusion

Overall, the research demonstrated that tropical ethnic leafy vegetable Malabar spinach has potential to successfully grow under Canadian maritime climatic conditions. Thus, local production gives food security to immigrants in Canada and reduces import of vegetable from other countries as well. The study of N rate alone revealed that medium N rate was optimum to produce maximum growth and yield of Malabar spinach under greenhouse environmental conditions. Results of the field experiment showed that under low temperature condition low N rate would help to significantly increase plant growth and yield. Under high temperature conditions there was no significant

differences between growth and yield of the control and the N applied plants. Planting date did not show any effect on total phenolic and total carotenoid content of Malabar spinach whereas N rate showed little effect on these chemicals. Nitrate content found in Malabar spinach was very low regardless of N rate applied. Thus, the research met the objectives of this study.

6.3. Recommendations for Future Study

This research study provided information about the suitable application rate of N for the cultivation of Malabar spinach under both greenhouse and field conditions. It also provided the information about Malabar spinach growth, yield and chemical composition responses to different planting dates. However, there are many challenges that needs to be overcome for the successful cultivation of Malabar spinach in Atlantic Canada. The following are recommended for future research prior to recommendation of N rate and planting date:

1. further investigation into the production potential of Malabar spinach is required in order to recommend for commercial cultivation.
2. identification of major pests and diseases and their management practices.
3. shelf life and storage effects on the quality of Malabar spinach.

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