The Chemical Composition of Baby Spinach (*Spinacia oleracea* L.) as Affected by Nitrogen, Phosphorus and Potassium nutrition

by

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DECLARATION

I hereby declare that the work that is being presented in this thesis **"THE CHEMICAL COMPOSITION OF BABY SPINACH (*Spinacia oleracea L.*) AS AFFECTED BY NITROGEN, PHOSPHORUS and POTASSIUM NUTRITION"** is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references. This thesis was subjected to turn-it in as per University policy.

____________________________ ________________________

SIGNATURE **DATE**

(MS BO ZIKALALA)

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LIST OF ABBREVIATIONS

ADI – Acceptable Daily Intake

ANOVA – Analysis of Variance

AOAC – Association of Official Analytical Chemists

ARC-VOPI – Agricultural Research Institute-Vegetable and Ornamental Plant Institute

AWCOC – Atteridgeville West Church of Christ

CBSs – Carbon Based Secondary Compounds

CDV – Cardiovascular disease

C - Celsius

Cv. – Cultivar

Cm^2 – Square Centimetre

CNB – Carbon/Nutrient Balance

CO_2 – Carbon dioxide

Fe – Iron

FAO – Food and Agriculture Organization

G – Gram

GDB – Growth Differentiation Balance

GLM – General Linear Model

GPS – Geographic Positioning System

HPLC – High-Performance Liquid Chromatography

K – Potassium

$Kg \cdot ha^{-1}$ – Kilogram per hector

Km – Kilometre

L – Linear

LAN – Limestone Ammonium Nitrate

Mg – Milligram

Mg - Magnesium

MS – Mean of Squares

MMOL.M^2-S – Millimol per meter square per second

NM – Nanomoles

N – Nitrogen

NO_3^- – Nitrate

Ns – Non-Significant

P – Phosphorus

PCM – Protein Competition Mode

Q - Quadratic

RCBD – Randomized complete block design

SAS – Statistical Analysis System

Se - Selenium

SOV – Source of Variance

SS – Sum of Squares

USA – Unites States of America

WHO – World Health Organization

Zn – Zinc

ABSTRACT

Baby spinach (*Spinacia oleracea* L.) is considered to be the one of the extremely nutritious vegetables, rich both in phytochemicals and core nutrients. Nowadays, phytochemicals in plants are raising interest in consumers for their roles in the maintenance of human health. Variation in content of bioactive compounds and core nutrients is the main concern in vegetable production. Factors such as cultural practices specially fertilization, may affect the nutritional and medicinal properties of the plants

Therefore, three parallel trials for NPK to investigate the response of baby spinach leaves to nitrogen (N), phosphorus (P) and potassium (K) on chemical composition were conducted, with treatments arranged as follows: 0, 45, 75, 105, 120 kg·ha^{-1} N and P and 0, 60, 85, 106, 127, 148 kg·ha^{-1} K in a randomized complete block design (RCBD) with four replications. The results demonstrated that, application of nitrogenous, phosphorus, potassium fertilizers significantly increased the total phenolic content, total antioxidant activity, total flavonoid content and vitamin C while magnesium, iron, zinc and selenium did not exhibit significant response to all treatments applied. The increase in concentrations on total phenolic content, total antioxidant activity, total flavonoid content and vitamin C was observed, reaching maximum at 45 kg·ha^{-1} N, 75 kg·ha^{-1} P and 85 kg·ha^{-1} K. The optimum rates of 45 kg·ha^{-1} N, 75 kg·ha^{-1} P, 85 kg·ha^{-1} K were then used to formulate the NPK treatment combinations as follows: 0, 30: 30: 40, 45:45:60, 60:60:70, 75:75:90 kg·ha^{-1}, arranged in a RCBD with three replicates. The results showed that total phenolic content, total antioxidant activity , total flavonoid content and vitamin C reached maximum in baby spinach leaves at N45:P45:K60 kg·ha^{-1}.

Keywords: baby spinach, bioactive compounds, minerals, NPK nutrition, concentrations, variations, phytochemicals, fertilizer, response, quality.

CHAPTER 1

1. RESEARCH ON BABY SPINACH

1.1 BACKGROUND

Spinach is a leafy and extremely nutritious vegetable, rich both in core nutrients and phytochemicals. It is a vegetable that is provided fresh, frozen or canned to the consumer. Spinach harvested after a shorter growth period than normal is called baby spinach, and is marketed fresh to the consumer. This is a fairly new product that has turned out to be popular in recent years because of its nutritional value (Hedges and Lister, 2007). Its nutrients comprise a range of vitamins and minerals, as well as phytochemicals. The major micronutrients in spinach are vitamins A (from β-carotene), C, K and folate, and the minerals, calcium, iron and potassium. The phytochemicals of most importance are the carotenoids, flavonoids, β-carotene, lutein and zeaxanthin and phenolic compounds (Bergquist, 2006). A number of studies have shown spinach to have strong antioxidant activity and high levels of antioxidant compounds such as phenolics and carotenoids (Hedges and Lister, 2007).

Agronomic practices such as chemical fertilizers have made major contributions to improve crop yields and food nutrition (Fageria, 2009; Wang *et al*., 2008). There are many contributing factors in terms of fertilizer application that can influence the effectiveness of fertilizer such as the application method, application timing and the rate of application. All these factors must be investigated before fertilizer application recommendations to ensure effectiveness and to prevent problems associated with fertilizers. Problems such as under application and over application of fertilizers are seen to be challenging. On the other hand, excessive fertilizer application can have detrimental consequences. It can have adverse environmental effects on water quality, leaching, and runoff (Heckman, 2007; Heckman *et al*., 2003; Manotti *et al*., 1994; Sims, 1998; Sims *et al*., 1998). Consequently, it is imperative to investigate and determine fertilizer application rates that are beneficial and maximize the yield while minimizing environmental contamination (Fontes *et al*., 1997; Heckman *et al*., 2003). The efficient use of fertilizers and optimal fertilizer management of nitrogen, phosphorus and potassium are necessary to minimize production cost and to improve yield and quality (Fageria, 2009).

Some studies are available on the identification of bioactive compounds in fruits and vegetables, pre-harvest and postharvest factors. However, information on the effect of mineral fertilization on nutritional quality of baby spinach is scarce. As the consumption of fresh-cut spinach increases, it is important to investigate the good management practices, specifically fertilization and its effects on quality of baby spinach. The consumption of spinach is increasing because people are becoming health cautious and spinach is regarded as one of the healthy vegetables which is rich in nutrients. The purpose of the present study was to investigate and quantify the variations on different bioactive compounds, namely, antioxidants, flavonoids, total phenolic content and carotenoids, as well as the trace elements (magnesium, zinc, iron and selenium), in baby spinach with reference to mineral nutrition.

1.2 RESEARCH PROBLEM

Spinach is an important agricultural crop, not only because of its economic importance, but also for the nutritional values of its leaves, mainly due to the fact that they are an excellent source of nutrients and phytochemicals. Spinach is increasingly becoming important in health because of its micronutrients and phytochemicals. It has an additional advantage of being low in calories, which is very important in weight management. Therefore, it is becoming a food of choice to many people because of its nutritional importance. Nowadays, phytochemicals and antioxidants in plants are raising interest in consumers for their roles in maintaining human health. Phenolics and flavonoids are known for their health promoting properties due to protective effects against cardiovascular disease, cancers and other diseases (Kaur and Kapoor, 2001; Sardas, 2003)

The intake of these compounds in foods is an important health protecting factor. They have been recognized as being beneficial for preventing widespread human diseases, including cancer and cardiovascular diseases, when taken daily in adequate amounts (Kaur and Kapoor, 2001; Sardas, 2003). It is generally known that environmental factors and agricultural techniques have an effect on vegetable and fruit quality. In particular, mineral fertilization influences antioxidant composition in some fruits and vegetables (Jeppsson, 2000; Kaur and Kapoor, 2001; Kopsell *et al*., 2006; Kemal *et al*., 2007). Some studies are available on the effect of postharvest storage and processing on the antioxidant constituents (Flavonoids and vitamin C) of Fresh-Cut spinach (Bergquist, 2006). However, information on the effect of mineral fertilization on nutritional quality of spinach is scarce. Variable content of bioactive compounds and minerals is the main problem in the production of vegetables, due to many factors especially fertilization. Consequently, the nutritional and medicinal value of vegetables may be affected. Producers often apply large quantities of fertilizer in an attempt to maximize yield and advance growth, which can result in the accumulation of nitrate in case of excessive nitrogen, reducing the quality of some vegetables. There are no reports about the content of bioactive compounds and minerals in baby spinach grown on the principles of best management practices in South Africa.

Currently the demand of baby spinach exceeds the supply and the applications of 22-45Nkg/ha, 22-45Pkg/ha and 63-138Kkg/ha have been reported in California, USA. These are the application rates from the growers since there is no scientific validation of these rates. Limited studies are available on the effect of nutrients on bioactive compounds of baby spinach and data that provide recommended rates such as N, P and K on quality have not been well established under South African conditions.

Therefore, it was important to investigate the variation in concentrations of bioactive compounds (antioxidants, flavonoids, carotenoids and total polyphenols) and trace elements (magnesium, iron, zinc and selenium) in baby spinach with reference to mineral nutrition. The main aim of this study was to investigate the effect of fertilization (N, P and K) baby spinach on their bioactive compounds. Additionally, any information on the physicochemical properties and bioactive compounds of baby spinach will provide a knowledge base that may be of some benefit to the production of baby spinach in South Africa.

1.3 MOTIVATION FOR MY RESEARCH

Spinach is one of the world's healthiest vegetables. Rich in vitamins and minerals, it is also concentrated in health promoting phytonutrients such as carotenoids (beta-carotene, lutein, and zeaxanthin) and flavonoids to provide powerful antioxidant protection (Mehta and Belemkar, 2014). Leafy green vegetables such as spinach provide more nutrients than any other food, when compared calorie for calorie (Longnecker e*t al*., 1997). Its nutrients include a range of vitamins and minerals (micronutrients), which can prevent deficiency diseases and are essential for normal physiological function, as well as phytochemicals thought to help prevent chronic health problems such as cancer and heart disease, as well as other health problems associated with ageing (Hedges and Lister, 2007).

In recent years, considerable attention has been directed towards identifying natural antioxidants, namely those plant-derived that may be used for human consumption regarding health promotion and disease prevention. It is vital to determine a method of how we could grow healthy vegetables vegetable so that human body could have a higher intake of health promoting bioactive compounds. This fact is very important in these days when people are exposed to lot of health related challenges. This study addresses such areas where the effect of mineral fertilization can be related to the concentration of health promoting phytochemicals.

One of the advantages of spinach is that it is readily available, just about all over the world and is easy to prepare. What is more interesting about spinach, it is easy to find in the market or easy to grow and prepare. With its fine taste, baby spinach is a versatile food that can be easily included into a range of dishes. It can be eaten raw in a salad or it can be cooked (steamed or boiled) and eaten as a dish on its own or added to soups and other dishes. Unlike other vegetables, it is harvested after a short period after planting. Over the years, ready to use fresh-cut spinach has become more popular as one of the important dietary vegetable (McGill *et al*., 1966; Burgheimer *et al*., 1967; Izumi *et al*., 1997).

Baby spinach was chosen for this study for two main reasons. It is becoming an increasingly popular product in South Africa and elsewhere in the world. Spinach is known to be a healthy product and contains relatively high concentrations of bioactive compounds (USDA, 2005). In addition, baby spinach has the advantage of a short culture time and shelf life, making it an excellent model crop. The objective of the study was to investigate the response of chemical composition and trace elements in baby spinach to fertilization with N, P, and K nutrition.

1.4 AIM OF THE STUDY

The aim of this study was to investigate and determine the response of total phenolic content, total antioxidant activity, total carotenoid content, total flavonoid content and vitamin C as well as minerals to fertilization with nitrogen (N), Phosphorus (P) and potassium (K) nutrition.

1.5 OBJECTIVE OF THE STUDY

1.5.1 To determine the effect of different rates/levels of application of N, P, and K fertilizers on chemical composition and minerals in baby spinach.

1.5.2 To determine the interaction effects of NPK on the bioactive compounds in baby spinach.

1.6 HYPOTHESES

1.6.1 Rate of applications of fertilizer N, P and K have no influence on the bioactive compounds in baby spinach.

1.6.2 No interaction effects on bioactive compounds in baby spinach after NPK application.

1.7 SCOPE AND LIMITATIONS OF THE RESEARCH

Given the research aim and objectives of the study delineated above, the scope of the study entailed three aspects – rate of applications of fertilizer N, P and K, the variation in bioactive compounds in baby spinach as well the combined effect of N, P and K on the bioactive compounds.

However, the study had a set of limitations inherent in the topic investigated. Firstly, not all varieties of baby spinach could be investigated for this research, given the nature of the research methods used and the time-frames selected. Secondly, the study was conducted in a protected environment and the effect of this was not taken into consideration in this study. Lastly, only one method of fertilizer application, side dressing was used in this study.

1.8 ETHICAL CLEARANCE

No ethical clearance of this study is required (Appendix C).

1.9 ORGANISATION OF THE THESIS

The thesis is presented in five chapters. Chapter 1 provides the research background on baby spinach, the importance and research problem of the study. An outline of brief research motivation, aim and objectives, scope and limitations of the study is also discussed. Chapter 2 consists of a review of the relevant literature pertaining to baby spinach. Chapter 3 provides a brief description of study areas, research design and methods, as well as data collection and analyses. Chapter 4 deals with set of research findings, with particular reference to the mineral nutrition and bioactive compounds in baby spinach. The chapter details the analyses and discussion of the variation in concentrations of bioactive compounds in baby spinach. The thesis ends with Chapter 5 which gives a summary of research findings, concluding remarks and recommendations which set goals for further research.

CHAPTER 2

2. LITERATURE REVIEW ON MINERAL NUTRITION AND QUALITY OF BABY SPINACH

2.1 INTRODUCTION

This chapter outlines and reviews literature. The review of literature includes the botany of baby spinach, chemical composition of baby spinach, health benefits as well as mineral nutrition of baby spinach. To end the chapter, a brief summary of these sections is also provided.

2.2 THE HISTORY AND BOTANY BABY SPINACH

2.2.1 Origin and distribution

Spinach originates from central and south-western Asia (Boswell, 2010). It is thought that it was first cultivated in ancient Persia (Iran) and eventually into Africa. From there it spread to Europe and now it is widely grown all over the world. (Asai *et al.,* 2004; Boswell, 2010). It was probably introduced into Europe during the Middle Ages; and brought to North America by European settlers (Asai *et al.,* 2004).

2.2.2 Botanical Description of baby spinach

Spinach (*Spinacia oleracea*) is an edible flowering plant in the family Amaranthaceae (LeStrange *et al*., 1999). Common spinach, *Spinacia oleracea*, was long considered to be in the Chenopodiaceae family, but in 2003, the Chenopodiaceae family was combined with the Amaranthaceae family under the family name 'Amaranthaceae' in the order Caryophyllales. Within the Amaranthaceae family, Amaranthoideae and Chenopodioideae are now subfamilies, for the amaranths and the chenopods, respectively. It is an annual plant, which grows to a height of up to 30 cm (Boswell, 2010).

Spinach is dioecious; plants generally produce either male (staminate) or female (pistillate) flowers but only one sex is to be found on any one plant so both male and female plants must be grown if seed is required and are pollinated by wind. The plant is not self-fertile (Asai *et al.,* 2004). Baby spinach is harvested after a shorter growth period and sown at closer density than regular spinach and thus the leaves are smaller, hence the name. Spinach is a long-day plant that is prone to bolting during the summer (Bergquist, 2006). The bolting tendency varies between cultivars, and cultivars that are less prone to bolting could be used during the earlier part of the summer. For baby spinach, bolting is often not a problem because the leaves are harvested early (Respondek and Zvalo, 2008).

2.2.3 Types of spinach

A distinction can be made between older varieties of spinach and more modern ones. Older varieties tend to bolt too early in warm conditions. Newer varieties tend to grow more rapidly, but have less of an inclination to run up to seed (Rolland and Sherman, 2006). The older varieties have narrower leaves and tend to have a stronger and bitterer taste. Most new varieties have broader leaves and round seeds (Rolland and Sherman, 2006).

Spinach cultivars are generally categorized according to the leaf blade variations and are classified as savoy have wrinkled leaves, semi-savoy have slightly crinkled leaves and flat or smooth leaved lacks wrinkles and are flat (Grieve and Grieve, 1971; Respondek and Zvalo, 2008, LeStrange *et al*., 1999).

2.3 COMPOSITION AND QUALITY OF BABY SPINACH

2.3.1 Micro nutrients

Spinach has high concentration of vitamin A, E, C, and K, and also folic and oxalic acids. Along with these chemicals are various minerals are present in the spinach such as magnesium, manganese, calcium, phosphorus, iron, zinc, copper and potash. (Mehta and Belemkar, 2014); Van der Walt *et al*., 2009). Spinach is an excellent source of chlorophyll, (needed for the production of vitamin A), riboflavin, sodium and potassium. Other nutrients present in smaller quantities include some B vitamins – thiamine (B1), riboflavin (B2) and B6 (Hedges and Lister, 2007).

2.3.1.1 Magnesium

Magnesium is one of the minerals present in the spinach (Mehta and Belemkar, 2014). It is part of the chlorophyll molecule existing in all green plants and is, consequently, important for photosynthesis and also helps to stimulate numerous plant enzymes required for development (Plaster, 2003; Gardiner and Miller, 2004). Magnesium deficiency symptoms appear first in older leaves as chlorosis (Acquaah, 2002). It was reported by Mengel and Kirby (1982) that shortage of magnesium impedes protein synthesis.

In humans, magnesium is an essential mineral needed for survival. The health advantages of magnesium are extensive. A number of studies have previously shown magnesium to have many health benefits. Magnesium deficiency is associated with poor growth and development, weak muscles and cardiac problems (McDowell, 1992).

2.3.1.2 Iron

Spinach is known to be a source of iron (Hedges and Lister, 2007). Iron is important for the synthesis of chlorophyll, which gives rise to the green colour of plants (Acquaah, 2002). Iron shortage can results in interveinal chlorosis of young leaves and chlorosis spreads to the older leaves as the severity of the deficiency increases (Jones, 2003). According to Anyoola *et al*. (2010), iron plays a key role in proper functioning of reproduction systems in both males and females.

2.3.1.3 Zinc

Zinc is an essential trace element present in spinach (Mehta and Belemkar, 2014). In plants, zinc helps with growth hormones, seed production and maturation, and starch formation (Brady and Weil, 2004). Zinc deficiency causes plant leaves to be severely reduced in size, while the internodes shorten to give a rosette appearance and the leaves become mottled (Acquaah, 2002; Gardiner and Miller, 2004).

In humans, zinc is found in all parts of the body: it is in organs, tissues, bones, fluids and cells. Muscles and bones contain most of the body's zinc (90%). The known health benefits of zinc include proper functioning of the immune system and physical growth (Hotz and Brown, 2004).

2.3.1.4 Selenium

Selenium is a trace element that is essential for human health in small amounts. Spinach is one of the green leafy vegetables which contain selenium as one of the minerals. Selenium plays a key role in metabolism. Selenium is significant in health because of its antioxidant properties. It has been suggested in some studies that selenium supplements may reduce the chances of prostate cancer (Hatfield *et al*., 2012).

2.3.2 Phytochemicals

Phytochemicals are compounds that are produced by plants. They are found in fruits, vegetables, grains, beans, and other plant parts. With most greens like spinach, when eaten raw it is loaded with a high vitamin and mineral content, but when cooked it releases many phytochemicals. Phytochemicals are natural plant organic compounds often called phytonutrients that are well known for potential health benefits. The phytochemicals of most importance in baby spinach are the carotenoids, β-carotene, lutein and zeaxanthin, along with phenolic compounds (Bergquist, 2006; Hedges and Lister, 2007; Mehta and Belemkar, 2014). Other phytochemicals include chlorophyll, glutathione, α-lipoic acid and betaine (Joseph *et al*., 2002).

2.3.2.1 Total antioxidant activity content

Antioxidants are naturally present in fruits and vegetables. They are chemicals often found naturally in plant foods, which can help protect body cells from being damaged by 'free radicals'. Free radicals are produced both naturally in our body and due to exposure to pollutants and result in cell damage (Cadenzas and Packer, 1996; Nicoli *et al*., 1999). Free radicals have been implicated in the aetiology of several major humans' ailments, including cancer, cardiovascular disease, neural disorders, diabetes and arthritis (Sies, 1996; Devasagayam *et al*., 2004).

The positive health effects of fruits and vegetables have been credited to the fairly high antioxidant concentration in fruits and vegetables (Ames *et al*., 1993; Rice - Evans and Miller, 1995). Numerous studies have examined the antioxidant activity of spinach and other vegetables. Cao *et al*. (1996) gave spinach an antioxidant score of 17 (based on ORAC scores using three different radicals), rating it third of the 22 vegetables examined. Similarly, Pellergrini *et al*. (2003) ranked spinach first; eighth and first respectively out of 31 popular vegetables. Another report (Wu *et al*., 2004) ranked spinach extremely highly for antioxidant activity.

2.3.2.2 Total phenolic content

Phenolic compounds are commonly found in both edible and non-edible plants. These compounds are of interest in the food industry because of their health benefit functions in food (Manach *et al*., 2004). Phenolic compounds increase the quality and nutritional value of food (Kahkönen *et al*., 1999). They are found in food sources such as fruits, herbs, vegetables, cereals, and other plant materials as shown in Table 1 (Manach *et al*., 2004).

Chemical structures range from quite simple compounds such as caffeic acid to highly polymerised substances such as tannins. There are many diverse groups of phenolics but the most common phenolics found in foods are generally phenolic acids, flavonoids, lignans, stilbenes, coumarins and tannins (Harbourne, 1993). Phenolic compounds believed to be potentially protective factors against cancer and heart diseases, in part because of their potent antioxidative properties and their ubiquity in a wide range of commonly consumed foods of plant origin (Mehta and Belemkar, 2014).

Table 1: Polyphenol content in vegetables (Source**:** Manach *et al.,* (2004).

Polyphenolic content	**Source**	**By wt or vol** *mg· kg*$^{-1}$*fresh wt (or mg· L*$^{-1}$*)*
Flavonols	Yellow onion	350–1200
Quercetin	Curly kale	300–600
Kaempferol	Leek	30–225
Myricetin	Cherry tomato	15–200
	Broccoli	40–100
	Beans, green or white	10–50
	Tomato	2–15
Flavones	Parsley	240–1850
Apigenin	Celery	20–140
Luteolin	Capsicum pepper	5–10
Daidzein	Soybeans, boiled	200–900
Catechin	Beans	350– 550
Sinapic acid	Potato	100–190

2.3.2.3 Total flavonoid content

Flavonoids are found in grains, vegetables, and fruits. Spinach is very rich in the flavonoids. Various flavonoids are reported to be present in spinach (Mehta and Belemkar, 2014). The concentration of flavonoids in vegetables and other plants differs depending on influences such as genotype, environmental growing conditions, growth stage, postharvest handling, and storage conditions (Patil *et al*., 1995, Howard *et al*., 2002). These factors affect both total flavonoid concentration and the composition of flavonoids may consequently result in variation in flavonoid content. According to Fico *et al*. (2000); Vogt and Gulz (1994), the concentration in flavonoids and composition may also change during plant growth. According to Bergquist (2007), the bioavailability of flavonoids is most probably lower than that of some other antioxidants, such as ascorbic acid. Some studies reported that a high dietary intake of flavonoids is related to a lower risk of coronary heart disease (Hertog *et al*., 1995; Knekt *et al*., 1996) dementia (Commenges *et al*., 2000) and some form of cancer (Neuhouser, 2004). According to Fico *et al*. (2000) and Vogt and Gulz (1994) flavonoid concentration and composition may also change during plant growth.

Flavonols

Flavonols are the most abundant flavonoids in foods, and the main representatives are kaempferol and quercetin (Manach *et al*., 2005). Considered as the most abundant dietary flavonol, quercetin is a potent antioxidant because it has all the right structural features for free radical scavenging activity. According to Manach *et al.* (2004), the richest sources are onions (up to 1.2 $g \cdot kg^{-1}$ fresh weight), curly kale, leeks, broccoli, and blueberries.

Flavones

In fruit and vegetable flavones are much less common than flavonols. Parsley and celery are identified as the richest sources by Manach *et al*. (2005). Cereals such as millet and wheat contain C-glycosides of flavones (Boyle *et al*., 2000 and Erlund *et al*., 2000). Nielsen *et al*. (2003) reported large quantities had been identified on the skin of citrus fruit.

Flavanones

In human foods, flavanones are found in tomatoes and certain aromatic plants such as mint, but they are present in high concentrations only in citrus fruit. The main aglycones (the non-sugar component that results from hydrolysis of glycoside flavanones) are naringenin in grapefruit, hesperetin in oranges, and eriodictyol in lemons (Hertog *et al*., 1993).

Isoflavones

Isoflavones are provided only by soybean-derived products. They are found almost exclusively in leguminous plants. Soya and its processed products are the major sources of isoflavones in the human diet (Hollman *et al*., 1996; Moon *et al*., 2000).

Flavanols

Flavanols are present in both the monomer form (catechins) and the polymer form (proanthocyanidins). Catechins are found in many types of fruit such as apricots, which are the richest source (Manach *et al*., 2005). Catechin and epicatechin are the main flavanols in fruit, whereas gallocatechin, epigallocatechin, and epigallocatechingallate are found in certain seeds of leguminous plants, in grapes, and more importantly in tea (Manach *et al*., 1998; Wittig *et al*., 2001).

2.3.2.4 Total carotenoid content

Spinach shows presence of different carotenoids like lutein, β-carotene, violaxanthin and 9'-(Z)-neoxanhin (Mehta and Belemkar, 2014). Spinach contains some of the largest amounts of lutein and zeaxanthin from vegetable sources. Although kale contains more, it is not commonly eaten and is therefore not a major food source. Studies have shown that these compounds are selectively accumulated in different parts of the eye, where they are by far the most abundant of the major carotenoids present (Hedges and Lister 2007). A study reported that they may be an important protection against age-related eye problems, particularly macular degeneration and the formation of cataracts (Stahl and Sies 2003; Mares-Perlman *et al*., 2002).

Their antioxidant activity has led to the notion that of carotenoids, particularly lutein, which is more widely dispersed in the body, they could protect against diseases such as cancer and cardiovascular disease as well as boosting the immune function (Hedges and Lister, 2007). In relation to cardiovascular disease, studies have found high serum levels of lutein and zeaxanthin to be associated with a reduced risk of coronary heart disease (Dwyer and Navab, 2001). Studies confirmed that the consumption of green leafy vegetables with carotenoids, especially lutein and zeaxanthin was associated with a reduced incidence of stroke (Joshipura *et al*., 1999).

2.3.2.5 Vitamin C

Research has shown that spinach leaves that look fully alive and vital have higher concentrations of vitamin C than spinach leaves that are pale in colour (Edenharder *et al.,* 2001). The benefits of vitamin C may include protection against immune system deficiencies, cardiovascular disease, prenatal health problems, eye disease, and even skin wrinkling. Vitamin C is important in protecting against endothelial dysfunction, high blood pressure, and the blood vessel changes that precede heart disease (Fotherby *et al.,* 2000).

2.4. OTHER PHYTOCHEMICALS IN BABY SPINACH

2.4.1 Glutathione

According to Pompella *et al*. (2003), glutathione is a significant antioxidant in plants, animals, fungi and some bacteria and archaea, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides. It is produced within the body and is rare in foods. One of major functions of glutathione is to protect DNA from oxidation, but it also detoxifies carcinogens, boosts the immune system, supports liver health and reduces inflammation (Joseph *et al*., 2002).

2.4.2 α-Lipoic acid

Like glutathione, α-lipoic acid is a vital antioxidant present in some food and mostly produced in the body. It is known to be important for energy metabolism and its antioxidant activity may protect against chronic diseases. In addition, it may improve memory (Joseph *et al*., 2002). According to Berkson (1998), it is a remarkable antioxidant that may slow ageing, repair liver damage and diminish the risk of heart disease, diabetes and cancer.

2.4.3 D-Glucaric acid

D-Glucaric acid (GA) is a nontoxic, natural compound (Walaszek *et al.,* 1997). It is believed that D-glucaric acid may lower blood cholesterol (Joseph *et al*., 2002).

2.4.4 Coenzyme Q10

Another endogenous compound, coenzyme Q10 is a critical component in energy metabolism. It also acts as an antioxidant in cell membranes and lipoproteins. This compound is present in foods such as meat, fish and oils, but spinach is one of the vegetable sources. It is thought that it may prevent cardiovascular disease by lowering levels of homocysteine, a compound associated with the development of heart disease (Joseph *et al.,* 2002).

2.4.5 Chlorophyll content

Not much is known about the health effects of chlorophyll, the primary photosynthetic pigment which causes the green colour in plants. There have been reports that it may protect against some form of cancer, as it binds to mutant DNA and prevents it from proliferating. As it is high in concentrations in so many edible plants, it may be associated with some protective effects observed with diets rich in green vegetables (Fahey *et al*., 2005).

2.5 THE HEALTH BENEFITS OF BABY SPINACH

2.5.1 Introduction

Fruit and vegetables contain a wide range of substances that are suggested to be part of these health-enhancing effects. Numerous studies have been conducted with regard to the health benefits of fruits and vegetables. There is strong evidence that a diet rich in fruits and vegetables has a positive result on human health, offering protection against degenerative diseases of ageing, such as heart disease, cardiovascular disease, Alzheimer’s disease, cataracts and several forms of cancer (Williamson, 1996; Liu *et al.,* 2000; Gandini *et al.,* 2000; Liu *et al.,* 2001; Joshipura *et al.,* 2001; Kang *et al*., 2005).

Fruits and vegetables are known for being rich in both micronutrients and phytochemicals (Hedges and Lister, 2007). Important micronutrients such as vitamins, minerals, fruit and vegetables contain other compounds that may have a positive result on human health. Phytochemicals such as carotenoids, flavonoids and other polyphenols, phenolic acids, glucosinolates, allylic sulphides, isothiocyanates, dietary fibres, phytosterols and monoterpenes promote human health (Lister, 1999; Kris-Etherton *et al.,* 2002).

These are the health benefits shown by different human and animal studies in relation to spinach consumption:

2.5.2 Protection against ageing and improves brain function

- Spinach protects brain function from premature ageing and slow old age's typical negative effects on mental capabilities by preventing the harmful effects of oxidation on brain (Wang *et al.,* 2005).
- One of the major health benefits of spinach is credited to carotenoid compounds, those are lutein and zeaxanthin, is that of protecting against eye diseases such as macular degeneration (gradual loss of central vision, associated with old age). More studies also indicated that spinach extracts and spinach compounds may delay or retard age-related loss of brain function, reduce the extent of post-ischaemic stroke damage to the brain. (Hedges and Lister, 2007; Christen *et al*., 2000; Morris, 2006).

2.5.3 Cancer-fighting antioxidants

- As spinach is rich in vitamin A and flavonoids, and those compounds which are known to help the body protected from lung and oral cavity cancers. Moreover, spinach leaves are believed to protect human body from cancers of colon and prostate (Byers and Perry, 1992). A study that was conducted on New England women who had less prevalence of breast cancer among those who ate spinach regularly. Moreover, a study with laboratory animals showed that spinach extracts can reduce skin cancer and have potential to slow down stomach cancer (Gates *et al.,* 2007; Longnecker *et al*., 1997; Williamson, 1996).

2.5.4 Promotion of heart health

- Spinach is an excellent promoter of cardiovascular health (Blomhoff, 2005). Regular consumption of spinach in the diet helps to protect human body from cardiovascular diseases. The antioxidant properties of spinach work collectively, to support good cardiovascular health by preventing the harmful oxidation of cholesterol (Blomhoff, 2005; Lucarini *et al*., 2006).

2.5.5 Assistance in fetal development

- Spinach contains amounts of many B-complex vitamins such as vitamin-B6 (pyridoxine), vitamin-B1 (thiamin), riboflavin, folates and niacin. Folates help prevent neural tube defects in the offspring (Bernhoft, 2010).

- It is also a good source of Omega-3 fatty acids. Enough consumption of Omega-3 fatty acids is vitally important for pregnant woman as they are critical building blocks of fetal brain and retina. Omega-3 fatty acids may also play a role in determining the length of gestation as well as prevent perinatal depression (Jensen, 2006).

2.5.6 Promotion of body health

- Spinach leaves are an excellent source of vitamin K. Vitamin K plays a vital role in strengthening the bone mass by promoting osteotrophic (bone building) activity in the bone (Shearer, 2009). Regular consumption of spinach in the diet helps prevent osteoporosis (weakness of bones), and iron-deficiency anemia (Bernhoft, 2010).
- Spinach is rich in vitamin C which helps the body to develop resistance against infectious agents and scavenge harmful oxygen-free radicals. It also rich in minerals such as potassium, manganese, magnesium, copper, iron and zinc. Potassium is an important component of cell and body fluids which help to control heart rate and blood pressure. Manganese and copper are used by the body as co-factors for the antioxidant enzyme, superoxide dismutase (McDowell, 1992). Copper is required for producing red blood cells. Zinc is a co-factor for many enzymes that regulate growth and development, sperm generation, digestion and nucleic acid synthesis. Magnesium in spinach works

towards healthy blood pressure levels. Iron is an important trace element required by the human body for red blood cell production and is a co-factor for oxidation-reduction enzyme, cytochrome-oxidase during cellular metabolism (Jensen, 2006; Hotz and Brown, 2004).

2.6. THE VARIATIONS IN CONCENTRATION OF BIOACTIVE COMPOUNDS

2.6.1 Introduction

The variations in concentration of bioactive compounds in fruits and vegetables have been observed in numerous studies. The concentration of bioactive compounds in fruits and vegetables are definitely changing and are influenced by numerous pre- and postharvest factors (Bergquist, 2006). These factors include cultivar, soil type, fertilization, irrigation, the degree of maturity at harvest, harvesting, storage and method of processing (Hedges and Lister, 2007). Variations can be higher or lower concentrations of a certain compound as a result of these factors (Bergquist, 2006). The following are the factors affecting or have some influence on the concentrations of bioactive compounds in spinach.

2.6.2 Factors affecting concentrations of bioactive compounds in baby spinach leaves

2.6.2.1 Cultivar

The fact of inter-cultivar variation in general is well established. A study of eleven commercial lines and fifteen breeding lines showed large variations in antioxidant activity and phenolic content (Hedges and Lister, 2007). Variations were also notable by growing season, with significantly higher levels of antioxidant activity and phenolic content in over-winter spinach (Howard *et al*., 2002).

2.6.2.2 Genetic variation

Another study also showed phenolic content variation according to genotype, as well as level of maturity, with quantities significantly higher at the mid-maturity stage (Pandjaitan *et al*., 2005). Genetic factors thus have a large influence on the content of bioactive compounds in fruit and vegetables. Variation between cultivars of the same species may also be large (Mercadante and Rodriguez-Amaya, 1991; DuPont *et al.,* 2000; Howard *et al*., 2002). Ascorbic acid is found in vegetables in widely varying concentrations (Lee and Kader, 2000).

2.6.2.3 Climatic requirements

Climatic conditions have a strong effect on the concentration of bioactive compounds (Weston and Barth, 1997). Climatic factors differ with growing site, and during the season (Howard *et al*., 2002). Temperature, both in terms of average temperature and the extremes during the growth period, may influence the chemical composition (Weston and Barth 1997; Lefsrud *et al*., 2005).

2.6.2.4 Agronomic practices

Agronomic practices, such as nutrient and water availability, also have influence on the bioactive compounds (Weston and Barth, 1997). There are many contributing factors in terms of fertilizer application that can influence the concentrations of bioactive compounds. To predict the response of bioactive compounds to nitrogen fertilizer may be complicated. The results may show both increasing and decreasing concentrations due to fertilization. This may depend on how plant growth responds to the fertilisation (Mozafar, 1993). Nitrogen fertilisation may also enhance foliage, reduce light intensity reaching shaded parts of the plant and this may affect the concentration of the bioactive compounds (Mozafar, 1994; Lee and Kader, 2000).

2.6.2.5 Method of processing

Processing, such as freezing, influences the nutrients in spinach. The amounts of some nutrients such as vitamin C and folate are reduced with freezing, but others, such as β-carotene, lutein and zeaxanthin, are improved (Hedges and Ledger, 2007). Cooking has influence over nutrients in spinach. Cooking has both positive and negative effects on nutrients (Joseph *et al*., 2002). It can reduce vitamin C and folate, on the other hand it can increase the bioavailability of total carotenoid content like β-carotene and lutein and zeaxanthin. Light cooking or steaming is often recommended to enhance carotenoid bioavailability while minimising the loss of other nutrients (Joseph *et al*., 2002).

2.6.2.6 Harvesting factors

There is considerable variation in the concentration of bioactive compounds during growth and maturation of fruits and vegetables. The variation is most likely or obvious in fruit ripening, where the carotenoids or flavonoids provides the colour of the ripe fruit (Kalt, 2005).

Harvesting baby spinach leaves early, while they are slightly smaller than at conventional harvest, may give increased concentrations of bioactive compounds. Harvesting later than the current conventional time may give a higher yield of bioactive compounds, but the concentrations may not be improved although some compounds are likely to be higher at a later harvest (Bergquist, 2006).

Timing of harvest during the day is also of significance, as there may be great variation in the concentrations during the day, probably related to water content (when concentrations are given on a fresh weight basis) or to light intensity (Mozafar, 1994; Veit *et al*., 1996).

2.6.2.7 Storage

Storage environment after harvest may have a significant influence on the concentrations of bioactive compounds (Kalt, 2005). Storage not only affects the concentration of these compounds, but the concentration of these compounds may affect storability as well. Baby spinach is generally sold fresh in polypropylene bags and the maximum storage time is about 10 days (Lucier *et al.,* 2004; Bergquist, 2006).

Temperature plays an important role in storability and it should be managed well to enhance storability. Storability can be significantly enhanced by lowering temperatures, increasing humidity and by modifying the surrounding atmosphere. Temperature must be managed well during storage as it can result in deterioration of baby spinach (Wills *et al*., 1998). Appropriate temperature is crucial for keeping the quality of harvested fruits and vegetables, both in terms of appearance and biochemical composition. A lower temperature decreases metabolic rates and thereby slows down deterioration. However, very low temperatures may also impose problems, for example freezing reduces vitamin C in spinach. Recommended storage temperature is close to 0°C (Wills *et al*., 1998).

The storage period is also of major importance, since the concentrations change over time. Light conditions during storage generally affect concentrations of bioactive compounds as well. Lighted storage may also increase ascorbic acid production (Toledo *et al*., 2003). Carotenoids, on the other hand, may extremely decline if vegetables are stored in light (Kopas-Lane and Warthesen, 1995).

2.7 THE AGRONOMIC MANAGEMENT OF BABY SPINACH

2.7.1 Seeding/Planting

Spinach is commonly sown into rows spaced 20-30 cm apart. In recent years, growers have experimented with spinach being grown in rows 5 cm apart and seed spaced 5 cm apart in the row. Spinach is seeded on raised beds with 10-20 rows in the bed. A bed that is raised a few centimetres will aid in air and water drainage. For the baby spinach market, commercial growers have been experimenting with seeding in rows spaced 5 cm apart and seeds spaced 1-1.5 cm apart in the rows. 2-5 rows of spinach are grown on raised beds which could be 50-100 cm wide (Respondek and Zvalo, 2008).

2.7.2 Soils

Spinach grows well on a variety of soils, although fertile, sandy loams with high organic matter content are preferred. Spinach grows well on well drained sandy loams or loams high in organic matter. Soil sampling and testing must be done before planting and regularly to determine the soil pH. Spinach is not tolerant to acidic soils; therefore soil pH should be between 6 and 6.8. Spinach is not tolerant to acidic soils, therefore it is recommended to take samples regularly to ensure the right soil pH. Soils with good drainage and that warm up early in the season are recommended for spinach especially early and over wintered crops should be planted on such soils (SADAFF, 2010).

2.7.3 Irrigation

Baby spinach has a relatively shallow root system and thrives on frequent, short irrigations to maintain uniformly moist soil for maximum leaf production (Bergquist, 2006). Spinach requires a regular supply of moisture since it is a shallow rooted crop and should receive 25 mm of water every five days from rainfall or irrigation. The first five days of plant growth (germination and seedling emergence) are very moisture dependant and require 5 mm-12 mm of water. (Respondek and Zvalo, 2008; Joseph *et al.,* 2002).

2.7.4 Fertilization

Fertilization is one of the most practical and effective pre-harvest methods to regulate and increase yield and nutritional quality of crops for human consumption (Fallovo, 2009).

Recommendations for supplemental organic matter, inorganic fertilizer, lime or manure should be based on a soil test and a nutrient management plan. Nutrient management plans balance the crop requirements and nutrient availability with the aim to optimize crop yield and minimize ground water contamination, while improving soil productivity. If spinach is stressed by a lack of nutrients, growth is retarded and the plants are more prone to bolting (Respondek and Zvalo, 2008).

Mineral nutrients are very important in vegetable production to maximize yield and to enhance quality. Fourteen mineral nutrients known, and these are classified as either macronutrients or micronutrients based on their plant requirements. Six macronutrients: Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulphur (S), and these are often classified as ‘primary’ macronutrients, because deficiencies of N, P and K are more common than the ‘secondary’ macronutrients, Ca, Mg, and S. The micronutrients include boron (B), chlorine (Cl), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni) and zinc (Zn) (Wiedenhoeft, 2006).

2.7.4.1 Nitrogen

Nitrogen is used by the plant to produce leafy growth and formation of stems and branches. Plants most in need of nitrogen include grasses and leafy vegetables such as cabbage and spinach. Basically, the more leaf a plant produces, the higher its nitrogen requirement (Wiesler, 1998). Nitrogen supply is one of the major environmental factors that control plant mechanisms and is closely correlated to crop quality. The effects of N on spinach on the content of nitrate, oxalic acid, vitamin C and other antioxidants have been reported in previous studies (Elia *et al*., 1998; Zornoza and Gonzalez 1998; Logan *et al*., 1999). The management of nitrogen fertilizer supply is important, particularly to ensure the adequate levels for normal plant growth and the levels that are suitable for human consumption (Maereka *et al*., 2007) as per WHO standards.

Baby spinach is a short – season crop that is harvested when the crop is young. As a result, the nutrient uptake is relatively low. For instance, the nitrogen (N) content of spinach may vary from twenty pounds of nitrogen per acre (22 to 45 kg·ha^{-1}). Spinach is a medium – heavy nitrogen feeder (Hedges and Lister, 2007*).* Nitrogen encourages leaf growth so it is useful for lawns, houseplants, spinach or other leafy vegetables. Nitrogen supplied during growth greatly increases the size and quality of spinach. Good leaf coverage is also important for photosynthesis so virtually all plants need nitrogen but too much can make a plant soft (Cao *et al*., 1996*).*

Autumn application of nitrogen is not recommended due to the risk of nitrate leaching beyond the root zone by the winter rains. Small quantities of nitrogen, 20 pounds per acre (22 kg·ha^{-1}) are applied before planting or at planting, an additional top dress or water – run application of 20 to 30 pounds of nitrogen per acre (22 to 34 45kg·ha^{-1}) is generally sufficient for baby spinach production. Soil nitrate levels greater than twenty parts per million in the top 15 cm are adequate for crop growth (Williamson, 1996).

Increased additions of N usually result in increased yield of crop plants (Greenwood *et al*., 1980; Szwonek, 1986). However, toxicity from excessively high N concentrations is possible. Severe yield decrease was reported when cabbage (*Brassica oleracea* L. var. *Capitata*) was grown at elevated N rates (602 mg·N L^{-1} (Huett, 1989).

2.7.4.2 Phosphorus

Phosphorus is essential for seed germination and root development. It is needed particularly by young plants forming their root systems and by fruit and seed crops. Root vegetables such as carrots, swedes and turnips obviously need plentiful phosphorus to develop well (King *et al*., 2008; Nielsen *et al*., 2001).

Levels above 60 parts per million are adequate for spinach growth, for soils below this level, especially in winter, pre-plant applications of 20 to 40 pounds per acre of P_2O_5 (22 to 45 $kg \cdot ha^{-1}$) or applications of 20 pounds per acre of P_2O_5 at (22 to 45$kg \cdot ha^{-1}$) planting are recommended (Joseph *et al.,* 2002*).*

2.7.4.3 Potassium

Potassium is used in the process of building starches and sugars so is needed in vegetables and fruits. Potassium in the form of form of potash encourages flowering, fruiting and good colour (Bergquist, 2006). Potassium deficiency can results in decrease in both the number of leaves produced and the size of individual leaves. The reduction in both photosynthetic assimilates production and assimilate transport out of the leaves to the growing fruit significantly contributes to the undesirable consequences that shortages of potassium have on yield and quality determination (Pettigrew, 2008). Different nutrients and phytochemicals in fruits and vegetables, including potassium, may be independently or jointly responsible for an apparent reduction in cardiovascular disease risk (Ignarro *et al*., 2007).

2.7.4.4 Lime

On acidic soils, spinach has low germination, yellowing and browning of the leaf tips, the roots will burn and growth of the plant is slowed. If the soil pH is too high, chlorosis may result in leaves. Lime should be applied to maintain the soil pH in the range 6.5 to 6.8. Spinach is very sensitive to soil acidity, therefore it is recommended to test the soil regularly to ensure the maintenance of pH in the acceptable range (Respondek and Zvalo, 2008).

2.7.5 Harvest

Harvesting for baby spinach can begin as early as a month after sowing, few leaves from each plant can be harvested. Bigger leaves can be pinched off right at the base of the plant, leaving the smallest leaves for next harvest. Leaves can be harvested when they are 3-4 inches tall at 35 days after planting (USDA, 2005; Gandini *et al*., 2000). The entire plant can be harvested when it reaches maturity, just prior to bolting. The time of harvest is crucial as there may be variations in the concentrations on chemical composition during the day as result of pre-harvest factors such as water availability and light intensity (Mozafar, 1994). Harvested spinach can be kept in a moisture-retentive container in the refrigerator for as long 40-50 days (Gandini *et al*., 2000).

2.7.5.1 Postharvest handling

Spinach is fairly perishable and yellows when stored at higher than recommended temperatures. Spinach must be stored in low temperatures of 0°C-5°C (Lucier *et al.* (2004). Lower temperature has positive effect on ascorbic acid and carotenoids content (Kalt, 2005). The content of ascorbic acid in spinach rapidly decreases during storage at ambient temperature (Beuscher *et al.,* 1999). However, the main cause of postharvest losses is decay related with mechanical damage during harvest and postharvest processes. Spinach has a large surface-to-weight ratio and a very high respiration rate, it should be cooled rapidly to prevent excessive weight loss and wilting. Spinach is sensitive to ethylene (increases yellowing and may increase decay) and moderately sensitive to freezing injury after harvest (Pellegrini *et al*., 2003; Respondek and Zvalo, 2008).

Spinach can be stored for 10-14 days at a temperature of 0°C and a relative humidity of 95-100%. Wilting, yellowing of leaves and decay are likely to occur after 10-14 days of storage (Pellegrini *et al*., 2003). Spinach is very sensitive to ethylene and should not be stored or transported with apples, melons or tomatoes because accelerated yellowing may result (Respondek and Zvalo, 2008).

2.8 BRIEF SUMMARY OF THE LITERATURE REVIEW

Spinach is known for being nutritious vegetable, with wide range of vitamins and minerals. This review states very clearly the importance of baby spinach in terms of human health as it is rich in nutrients which can prevent deficiency diseases and are essential for normal physiological function, and phytochemicals which are believed to help prevent chronic health problems such as cancer and heart disease, as well as other health problems associated with ageing. Both minerals and phytochemicals are essential to promote good health.

The study discussed the agronomic management of baby spinach from planting to harvesting. Agronomic practices such as soil requirements, water availability and nutrition were conferred. It is crucial for these requirements to be adequate for the maximisation of yield and enhancement of quality.

Pre-harvest and post-harvest factors have effects on minerals and phytochemicals in vegetables. These factors may lead to lower or higher concentrations (Bergquist, 2006). Agronomic practices such as the selection of suitable planting material, field preparation, fertilizer application, weeding and irrigation have influence on phytochemicals in vegetables. Genetic factors, cultivar selection, harvesting and storage also have influence on the phytochemicals in baby spinach. All these factors and their influence on the phytochemicals in baby spinach were discussed. Good agricultural practices remain the key in the production of vegetables for the maximisation of yield and enhancement of quality. It is without a doubt that agronomic practices such as fertilization are important in relation to quality, and proper management of such factors is essential in vegetables.

Therefore, this present review serves as groundwork to a research to investigate and determine the effects of nitrogen, phosphorus and potassium fertilization on bioactive compounds (antioxidants, flavonoids, total phenolic content and carotenoids) of baby spinach. The importance of baby spinach in human health can never be over-emphasized; therefore it is crucial to investigate agronomic practices such as mineral nutrition (nitrogen, phosphorus and potassium fertilization) that can enhance the quality of baby spinach. This can contribute to South African food security and olericultural industry.

CHAPTER 3

3. DESCRIPTION OF STUDY AREA AND RESEARCH METHODOLOGY

3.1 INTRODUCTION

This section of thesis deals with the details of experiments which have been carried out. Chapter 3 provides a description of the study area, followed by a clarification of data collection methods and analyses techniques. The study employed quantitative research approach to address its objectives.

3.2 MATERIALS AND METHODS

3.2.1 Experimental site

The study was carried out at Agricultural Research Council - Vegetable and Ornamental Plant Institute (ARC-VOPI) in Roodeplaat farm, situated in the sourish mix of bushveld, 25 km north of central Pretoria, KwaMhlanga (R573) road; GPS coordinates: 25,56S;28,35E (Gauteng province, South Africa). The area is a relatively cool subtropical climate with summer rainfall and cold, dry winter.

3.2.2 Experimental site design and treatment details

3.2.2.1 Experimental site design

Baby spinach (*Spinacia oleracea* L.) cv. Ohio was used as a model crop in the studies reported in this thesis. Three trials were conducted to determine the optimum fertilizer rate(s). Seedbeds were prepared and filled with virgin red soil. A total of twelve seedbeds were prepared for all three trials. Each trial had five treatments, replicated four times. For each trial, four seedbeds were allocated and twenty plots were demarcated for planting. Each experimental plot size was 1.2m x 1m.

Timed sprinkler irrigation system was used for irrigation. All trials were planted twice at different times under a protected environment and planting of seedlings was done from February to March 2014 for first the 1st planting of trials and from April to June 2014 for the 2nd planting of trials. Spinach seeds were sown in seed trays filled with agro mix as a growing medium and later transplanted into beds. Thirty seedlings were transplanted into three rows on each plot with 20 cm between rows and 10 cm between plants. The 1st and 3rd rows were border plants, and the 2nd row was the net plot. Samples were collected only from the data plants as displayed below in Plate 1.

Plate 1: A bed of baby spinach demarcated into five different plots and five different treatments of N, P and K as shown in Table 2

The spinach was grown and harvested after 35 to 45 days. The leaves were harvested manually using sharp scissors by simply cutting the leaves at the stem as demonstrated in Plate 2. Each trial was harvested twice at 35 days and 45 days as last harvest. The outer oldest leaves were harvested first and the centre of the plant was harvested as those leaves matured. This method was used to allow it to re-sprout and give another partial harvest. After harvest, the samples were labelled accordingly, weighed for fresh mass and oven dried for 48 hours at 35°C.

Plate 2: Manually harvesting of baby spinach using sharp scissors

3.2.2.2 Treatment details

Three (N, P, and K) parallel trials were conducted under protected environment. Treatments consisted of 0, 45, 75, 105, 120 kg·ha^{-1} N; 0, 45, 75, 105, 120 kg·ha^{-1} P and 0, 60, 85, 106, 127, 148 kg·ha^{-1} K in a RCBD with five treatments replicated 4 times as shown in Table 1. Fertilizer sources applied were Limestone Ammonium Nitrate (LAN, N = 28%) for N trial, Superphosphate (P = 83%) for P trial and Potassium Chloride (K = 50%) for K trial. N, P and K fertilizers were applied as side dressing applications in the form of granules at two weeks after sowing.

Table 2: Treatment arrangement for Nitrogen (N), Phosphorus (P) and Potassium (K) trials

N P K trial treatments		
Applied Nitrogen (kg/ha)	Applied Phosphorus (kg/ha)	Applied Potassium (kg/ha)
0	0	0
45	45	60
75	75	85
105	105	127
120	120	148

[Z] 22-45N, P kg·ha^{-1}ha and 63 -138K kg·ha^{-1} recommended rates in California, USA, from growers without published scientific work.

Plate 3: Three parallel NPK trials layout

After the first experiment of N, P, K was conducted and completed as shown in Table 1, the results were analysed and the optimum rates of nitrogen, phosphorus, potassium were recorded. The maximum rates where the concentrations on bioactive compounds in baby spinach increased were used to conduct the second experiment of this study which was NPK treatments combinations. The trial consisted of five treatments and replicated four times. The treatment combinations were arranged as follows: 0, 30:30:48; 45:45:60; 60:60:78; 75:75:93.

3.2.3 Visual appearance

The variations in growth as a results of different levels of NPK treatments as shown in Plate 4.

Plate 4: Two plots of baby spinach displayed the variations in growth and leaves appearance in different NPK treatments

3.2.4 Chemical analyses of bioactive compounds

After harvest, the leaf samples were collected in paper bags, labelled accordingly, weighed for fresh mass and oven dried for 48 hours at 35°C, and the compounds were extracted using suitable solvents. Dry weights of the plants were taken prior to grinding the leaf samples in a laboratory mill. After grinding, these samples were placed in a dry place until the chemical determinations could be made. Analyses of the bioactive compounds were conducted using reversed-phase high-performance liquid chromatography.

3.2.4.1 Trace element content

The minerals, magnesium, iron, zinc and selenium were determined in a dilute solution of the ashed samples by atomic absorption spectrophotometer (3300 Perkin-Elmer) as described in Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC), edited by Horwitz (2000). The results were expressed on a dry weight basis.

3.2.4.2 Total phenolic content

About 15 g of finely ground leaf material was sieved. Total polyphenol concentrations were determined using the Folin-Ciocalteu the method by Waterman and Mole (1994) as described by Mudau *et al*. (2006).

3.2.4.3 Total carotenoid content

About 15 g of finely ground leaf material was sieved. For carotenoids gradient a reversed-phase HPLC elution was used, β-carotene for the carotenoids as described by Bergquist, *et al*. (2005). The detector used for quantification purposes and identification of carotenoids was a diode array detector. Total carotenoid content was described as mean ± standard deviation in mg 100 g^{-1} dry mass (Müller, 1997).

3.2.4.4 Total antioxidant activity

About 15 g of finely ground leaf material was sieved. For antioxidants, TEAC (Trolox equivalent antioxidant capacity technique was used as described by Miller *et al.* (1993).TEAC is a spectrophotometric technique that measures the relative ability of hydrogen-donating antioxidants to scavenge the ABTS+ radical cationchromogen in relation to that of Trolox, the water soluble vitamin E analogue which is used as an antioxidant standard. The ABTS+ which was produced by mixing equal volume of 8mM ABTS with 3mM potassium persulfates was prepared in distilled water and allowed to react in the dark for at least 12 hours at room temperature before use. The ABTS+ solution was diluted with a phosphate buffer solution (pH 7.4) mixed with 0.2 M of NaH_2PO_4, 0.2 M NaHPO4 and 150mM NaCl in 1 litre of distilled water, with pH adjustment using NaOH when necessary. A freshly-prepared solution was used for each analysis. The ABTS+ solution (2900µl) was added to the methanol extracts of baby spinach (100 µl) of Trolox in a test tube and mixed. Absorbencies reading (at 734nm) were taken after 30 minutes (for the samples) and 15 minutes (for the standard) of the initial mixing of the samples and standard, respectively. The results were expressed as µM Trolox equivalents /g of sample on dry weight basis.

3.2.4.5 Total flavonoid content

About 15 g of finely ground leaf material was sieved. Flavonoids were analyzed by reversed-phase HPLC, as described by Bergquist *et al*. (2005). For flavonoids gradient a reversed-phase HPLC elution was used. Flavonoids were identified using LC-MS/MS (liquid chromatography combined with mass spectrometry). Samples were extracted in triplicate under dim green light, using 60 mg plant material with 5mL 40% methanol. Before choice of solvent, different compositions of the extraction solution (40, 50 and 85% methanol or ethanol in water) were tested. The samples were shaken for 20 h at 4°C, 150 rpm and centrifuged at 10 000 × g at 4°C for 10 min. The supernatants were transferred to glass vials and stored at −80°C until analyzed by HPLC. Flavonoids concentration was quantified at 340nm using an external spiraeoside standard (Extrasynthese, Lyon, France). The flavonoids were identified by comparing retention times and absorption spectra with compounds previously identified by LC–MS/MS.

3.2.4.6 Vitamin C

Ascorbic acid (vitamin C) content was determined titremetrically by the method of Barakat *et al*. (1973). This method is as described by AOAC (1999). Ascorbic acid content was expressed as mg/100 g, dry weigh basis.

3.2.4.7 Method for N (nitrogen) determination

About 15 g of finely ground leaf material was sieved for N determinations on a Carlo Erba NA 1500 C/N/S Analyzer, using approximately 8 to 14 mg sample weighed into a tin foil container for each determination as described by Matejovic (1995); Jimenez and Ladha (1993). This method is a dry oxidation method generally known as the Dumas method. The compound chosen for the calibration standard was the ethyl ester of 4-Aminobenzoic acid, which contains 8,48% N. PeakNet software (Dionex Corporation, May 1998), with an external A/D interface (UI20 Universal Interface, Dionex) was used for data collection, peak integration, calibration and computation of concentrations (Dionex Corporation, 1998).

3.2.4.8 Sample extraction and determination of nitrate (and nitrite)

A method described by Matejovic (1995); Jimenez and Ladha (1993) was used for extraction and determination of nitrate (and nitrite). Distilled water was used to extract a sub-sample of the sample, using 0.2g sample to 50ml water and shaken on a mechanical shaker for 30 minutes, before filtering for sample extraction for nitrate (and nitrite). Determination of nitrate (and nitrite) was conducted by analysing the water extract solution by ion chromatography, which detects nitrate as well as most of the other major anions (nitrite, chloride, fluoride, sulphate etc.), by separating the anions on an ion exchange column and detecting them with a conductivity detector.

3.2.4.9 Computation of protein N and protein

The calculation of protein N was estimated using a method described by Matejovic (1995); Jimenez and Ladha (1993) where protein N was estimated by subtraction of the nitrate N from the total N.

3.2.4.10 Method for perchloric + nitric acid sample digestion

The method for perchloric + nitric acid sample digestion described by Zasoski and Burau 1977. 1g of sample was digested with 7ml HNO^3 (conc. nitric acid) and 3ml HClO4 (perchloric acid) at temperatures up to 200°C and brought to volume in a 100ml vol. flask

3.2.4.11 ICP-OES determination of P and K

Unpublished method developed by Mike Philpott at ARC-ISCW, based on the recommended procedures in the instrument manual (Liberty Series II, 1997). An aliquot of the digest solution was used for the ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometric) determination of P and K. The instrument used (Varian Liberty Series II) is a sequential instrument. The instrument was set up and operated according to the recommended procedures in the instrument manual.

3.2.5 Data collection

For all the treatments, at harvest (31 March 2014; 7, 11, 18 April 2014; 9, 23 May 2014; 27 June 2014 and 28 July 2014), fresh and dry shoot mass (g) weighed by Symmetry PR Precision Scale were recorded. Parameters recorded were total phenolic content, total antioxidant activity, total carotenoid content, total flavonoid content, vitamin C, magnesium, iron, zinc, selenium, total leaf tissue nitrogen, phosphorus and potassium.

3.2.6 Statistical analyses.

Data collected, were subjected to analysis of variance (ANOVA) using the GLM (general linear model) procedure of SAS, version 8.0. (SAS Institute, 1999). In all trials, treatment sums of squares were partitioned into linear and quadratic polynomial contrasts for total phenolic content, total antioxidant activity, total carotenoid content, total flavonoid content, vitamin C, magnesium, iron, zinc, selenium, total leaf tissue nitrogen, phosphorus, and potassium.

CHAPTER 4

4. RESEARCH RESULTS AND DISCUSSION

4.1 Research results

This results section presents the findings of the study in relation to the research objectives based on the collected and analysed data. In these experiments, chemical composition analysis was done for minerals (magnesium, iron, zinc and selenium) and bioactive compounds (total phenolic content, total carotenoid content, total flavonoid content, total antioxidant activity and vitamin C). The results show the variations in bioactive compounds of baby spinach in relation to different rates of applied nitrogen phosphorus and potassium fertilizers as well as the NPK treatment combinations.

4.2. Response of chemical composition of baby spinach to nitrogen nutrition

Table 3 indicates the variations in concentration on total phenolic content, total antioxidant, total carotenoid content and vitamin C, magnesium, iron, zinc and selenium concentrations in baby spinach as a result of nitrogen applied at different rates.

Table 3: Response of chemical concentrations in baby spinach to different levels of nitrogen (N) nutrition (dry weight basis)

Nitrogen Applied ($kg \cdot ha^{-1}$)	Magnesium (ppm)	Iron (ppm)	Zinc (ppm)	Selenium (ppm)	Total Phenols ($mg \cdot g^{-1}$)	Total Carotenoids ($mg \cdot g^{-1}$)	Total flavonoid content ($mg \cdot g^{-1}$)	Total Antioxidant Activity ($mg \cdot g^{-1}$)	Vitamin C ($mg \cdot g^{-1}$)	Protein N %	Total N%	Nitrate %	Leaf protein %*
Control	1.01a	0.91a	1.123a	0.001a	3.07b	0.89a	4.00b	0.49b	4.46b	1.34b	1.34b	0.02b	8.30b
45	1.01a	0.11a	1.010a	0.001a	8.10a	1.01a	8.10a	3.01a	10.9a	4.01a	4.01a	0.04b	25.0a
75	1.01a	0.91a	1.123a	0.001a	6.21a	1.50a	7.21a	2.13a	8.12a	4.26a	4.31a	0.23b	26.6a
85	1.01a	0.01a	0.010b	0.001a	6.10a	1.48a	7.10a	2.01a	7.12a	4.22a	4.34a	0.57b	26.3a
105	1.01a	0.91a	1.123a	0.001a	6.01a	1.39a	6.01a	2.00a	6.15a	4.22a	4.54a	1.33a	26.5a
120	1.02a	0.89a	1.021a	0.001a	5.90b	1.39a	6.01a	1.90b	6.10b	4.20a	4.60a	1.40a	26.2a
Significance	NS	NS	NS	NS	Q	NS	Q	Q	Q	Q	Q	Q	Q

Linear (L) or quadratic (Q) effects significant at P = 0.05 (*), 0.01 (**) or non-significant (NS)

Means with the same letter are not significantly different at 5% level of probability

4.2.1. Response of minerals (Magnesium, Iron, Zinc and Selenium) to nitrogen nutrition

Table 3 showed that different nitrogen levels did not have any significant effect on magnesium, iron, zinc and selenium concentrations of baby spinach leaves.

4.2.2 Response of total phenolic content to nitrogen nutrition

Results in Table 3 and Figure 3.1 show that Total phenolic content increased quadratically in response to nitrogen application. Total phenolic content level peaked at 45kg·ha^{-1}. The application of 45kg·ha^{-1} improved the total polyphenol content reaching maximum at 8.1 mg·g^{-1} (Table 3 and Figure 3.1). The total phenolic content increased from 0 to 45kg·ha^{-1}. The results showed that the total phenolic content of baby spinach deteriorated with increasing nitrogenous fertilizer rate ranges from 75 to120 kg·ha^{-1}. The difference between the highest (8.1 mg·g^{-1}) and lowest (3.07 mg·g^{-1}) mean value on Total phenolic content was 5.03 mg·g^{-1} on dry mass basis (Table 3 and Figure 3.1).

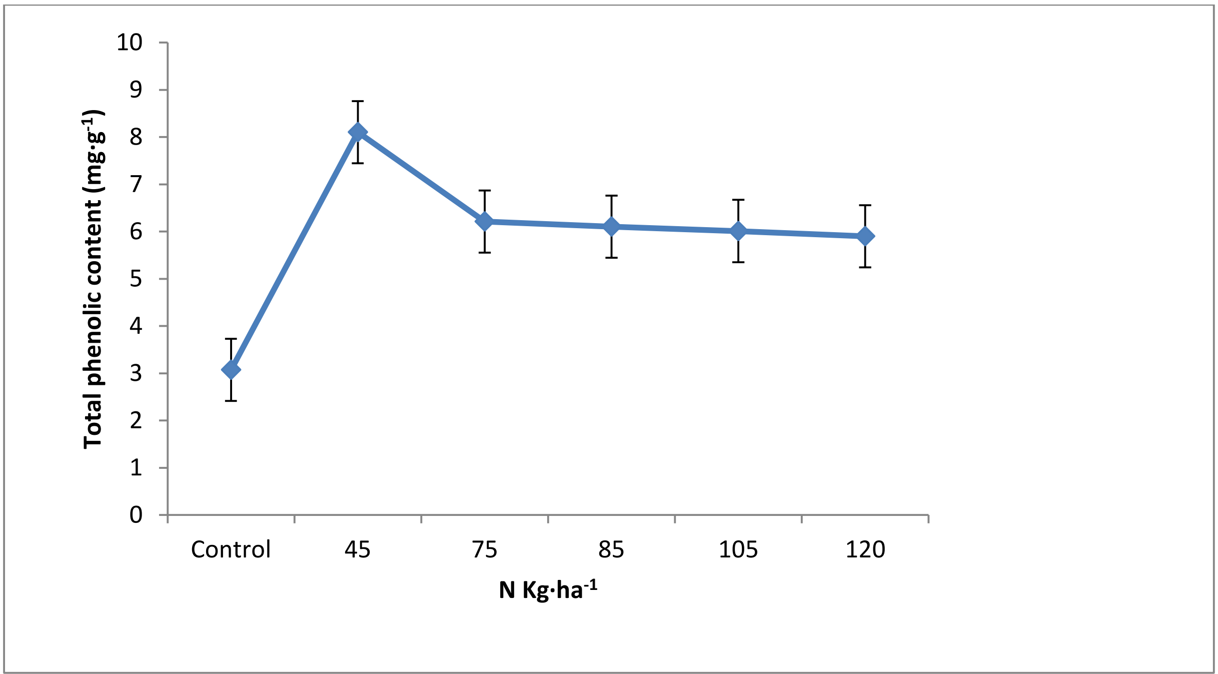

Figure 3.1: Total phenolic content concentrations of baby spinach at different rates of nitrogen application (dry weight basis)

4.2.3 Response of total carotenoid content to nitrogen nutrition

The results showed that different nitrogen levels applied did not have any significant effect on the carotenoids content of baby spinach leaves (Table 3). This suggests that there was no significant increase observed on the concentration of carotenoids regardless of the level of N applied.

4.2.4 Response of total flavonoid content to nitrogen nutrition

Concentration of total flavonoid content showed variations in baby spinach leaves (Table 3 and Figure 3.2). The lowest total flavonoid content in the baby spinach leaves concentration were 4.0 $mg \cdot g^{-1}$ and the highest concentration was 8.1 $mg \cdot g^{-1}$ (Table 3 and Figure 3.2). All treatments significantly improved total flavonoid content. The significant increase ranges from 4.0 $mg \cdot g^{-1}$ to 8.1 $mg \cdot g^{-1}$ after treatments application. The difference between the lowest and the highest concentration of total flavonoid content in baby spinach leaves was 4.1 $mg \cdot g^{-1}$ on dry mass basis. There were no differences between the N treatments.

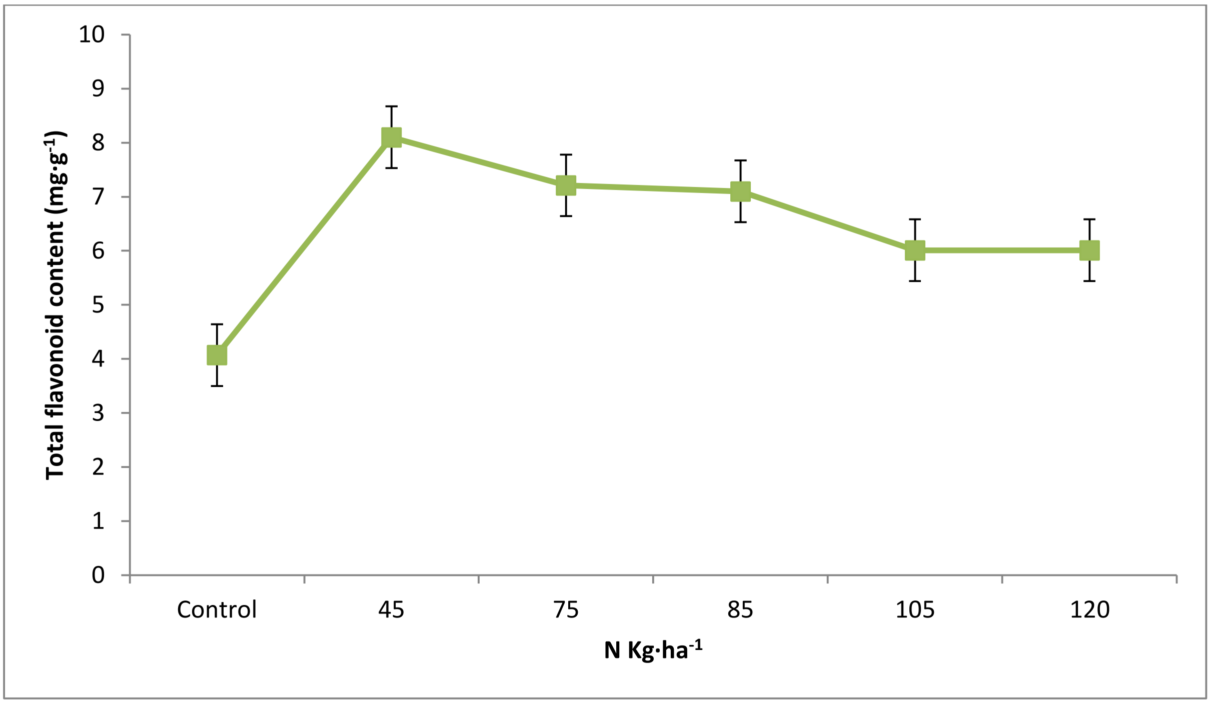

Figure 3.2: Total Flavonoid concentrations of baby spinach at different rates of nitrogen application (dry weight basis)

4.2.5 Response of total antioxidant activity to nitrogen nutrition

The application of 45kg·ha^{-1}N improved the total antioxidant activity content reaching 3.01 mg·g^{-1} (Table 3 and Figure 3.3). The total antioxidant activity content of plants under 45 kg·ha^{-1} was significantly higher compared to the control. The significant increase ranges from 0.49 mg·g^{-1} to 3.01 mg·g^{-1} after treatments application. The difference between the highest and lowest mean value on total antioxidant activity content was 3.52 mg·g^{-1} on dry mass basis (Table 3 and Figure 3.3).

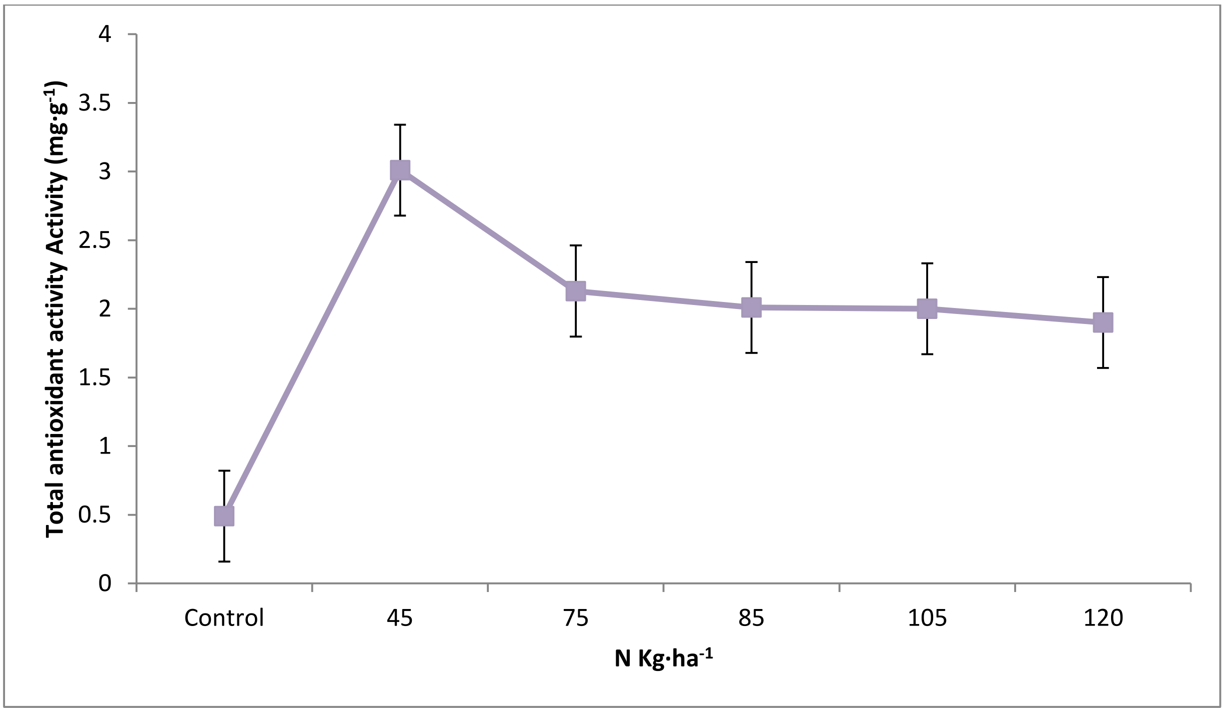

Figure 3.3: Total Antioxidant activity concentrations of baby spinach at different rates of nitrogen application (dry weight basis)

4.2.6 Response of Vitamin C to nitrogen nutrition

Results in Table 3 and Figure 3.4 showed that there was a variation in concentrations of vitamin C in baby spinach. Vitamin C was quadratically increased by application. Highest concentrations were at 45 kg·ha^{-1}. Vitamin C response to N occurred between 0 to 45 kg·ha^{-1}. The lowest vitamin C in the leaves concentrations was 4.46 mg·g^{-1} and the highest concentrations was 10.9 mg·g^{-1} (Table 3 and Figure 3.4). The difference between the highest and lowest mean value on total antioxidant activity content was 6.44 mg·g^{-1} on dry mass basis (Table 3 and Figure 3.4). The control and 120 kg·ha^{-1} had significantly lower vitamin C content than the other N treatments.

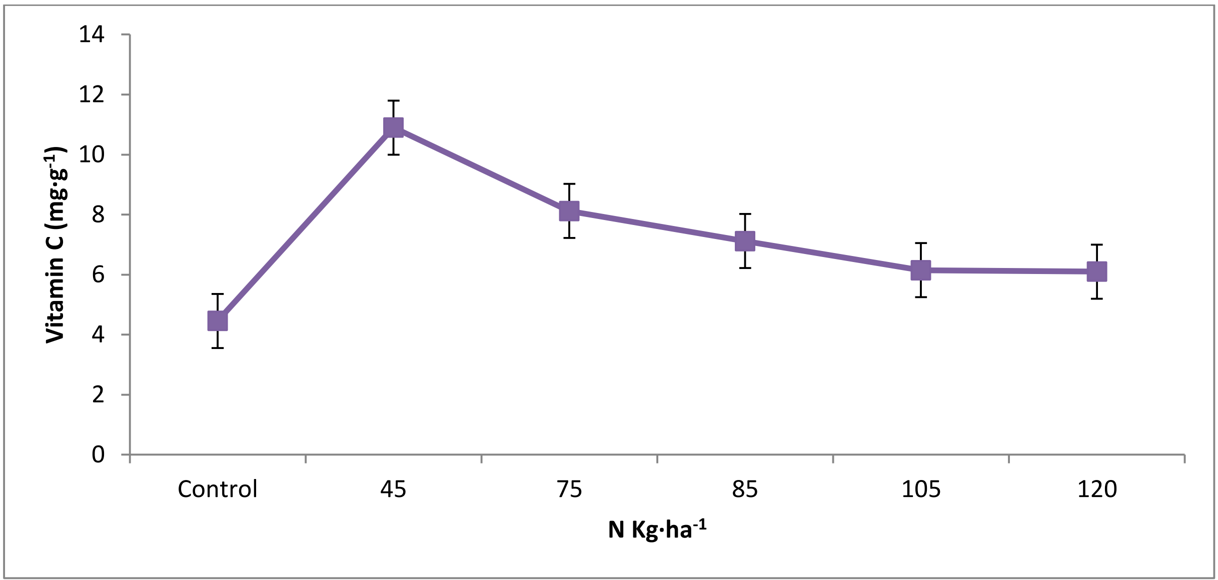

Figure 3.4: Vitamin C concentrations of baby spinach at different rates of nitrogen application (dry weight basis)

4.2.7 Percentage protein N

Percentage protein nitrogen quadratically increased with increasing nitrogen ranging from 1.34% to 4.26% (Table 3 and Figure 3.5). Nitrogen application significantly increased % protein N of baby spinach compared to control reaching maximum at 45 kg·ha^{-1}. The difference between the highest and lowest value was 2.92% (Table 3 and Figure 3.5).

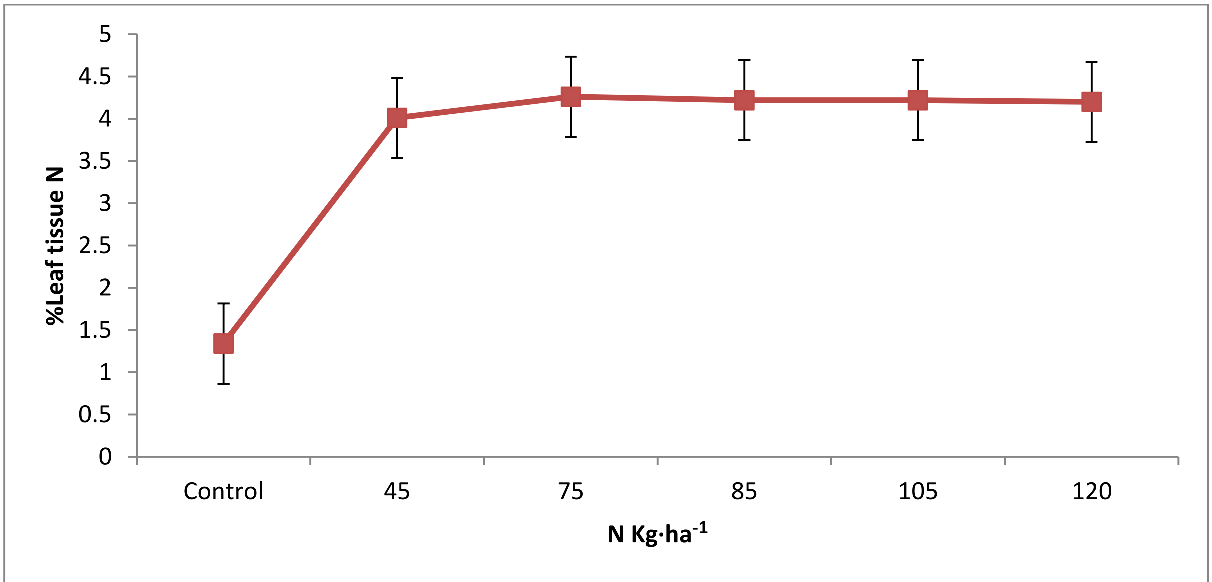

Figure 3.5: Leaf tissue nitrogen content in baby spinach

4.2.8 Percentage total N

The highest percentage total nitrogen of 4.60% was observed at 120 kg·ha^{-1}, causing a linear response with high rates of nitrogen applied. The difference between the highest value and lowest value was 3.26 (Table 3 and figure 3.6).

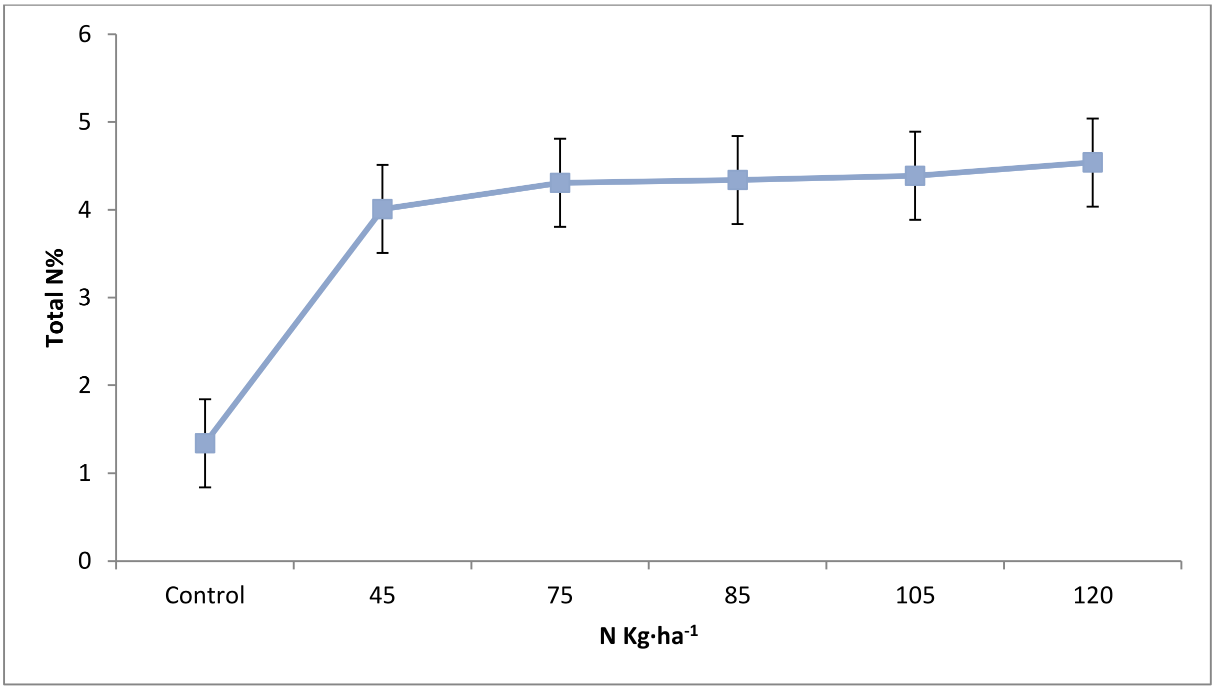

Figure 3.6: Total N% in baby spinach

4.2.9 Percentage nitrate

The results in Table 3 showed that there was a correlation between nitrogen applied and percentage leaf nitrate found in the leaves of baby spinach. Nitrate percentage in the baby spinach leaves increased with increasing nitrogen application reaching maximum of 1.40% at 120 kg·ha^{-1} (Table 3). The difference between the highest and lowest value is 1.309% (Table 3).

4.3 Response of chemical composition and minerals to phosphorus nutrition

Table 4 below indicates the variations in concentration on total phenolic content, total antioxidant activity, total carotenoid content and vitamin C, magnesium, iron, zinc and selenium in baby spinach as a result of phosphorus applied at different rates.

Table 4: Response of chemical concentrations in baby spinach to different levels of phosphorus (P) nutrition (dry weight basis)

Phosphorus Applied (kg·ha^{-1})	**Magnesium (ppm)**	**Iron (ppm)**	**Zinc (ppm)**	**Selenium (ppm)**	**Total Phenols (mg·g^{-1})**	**Total Carotenoids (mg·g^{-1})**	**Total flavonoid content (mg·g^{-1})**	**Total Antioxidant Activity (mg·g^{-1})**	**Vitamin C (mg·g^{-1})**	**% Leaf tissue P**	**Protein %***
Control	1.01a	0.91a	1.123a	0.221a	3.13b	0.89a	5.13b	3.50b	5.42b	0.526b	13.3c
45	1.01a	0.11a	1.010a	0.201a	8.10a	1.01a	9.32a	4.01a	9.90a	0.968a	21.3b
75	1.01a	0.81a	1.123a	0.211a	7.21a	1.60a	7.31a	2.93b	8.12a	0.948a	26.4a
85	1.01a	0.21a	0.010a	0.201a	5.10b	1.58a	7.41a	2.81b	7.32a	0.950a	26.3a
105	1.01a	0.91a	1.123a	0.221a	4.01b	1.49a	6.21b	2.5b	6.55b	1.115a	26.8a
120	1.01a	0.002a	1.021a	0.201a	4.90b	1.39a	6.01b	2.4b	6.40b	1.119a	26.9a
Significance		NS	NS	NS	NS	Q	Q	Q	Q	Q	Q

Linear (L) or quadratic (Q) effects significant at P = 0.05 (*), 0.01 (**) or NS

Means with the same letter are not significantly different at 5% level of probability

4.3.1 Response of minerals to phosphorus nutrition

Results in Table 4 showed that regardless of different levels of phosphorus applied, there was no significant effect on magnesium, iron, zinc and selenium concentrations of baby spinach.

4.3.2 Response of total phenolic content to phosphorus nutrition

Results in Table 4 and Figure 4.1 show that there was a quadratic increase of total phenolic content with application of phosphorus nutrition. In the P trial, total phenolic content reached their maximum at 45 kg·ha^{-1} with total phenolic content concentration of 8.10 mg·g^{-1}. Most of total phenolic content response to P occurred between 0 to 75 kg·ha^{-1}. The difference between the highest (8.10 mg·g^{-1}) and lowest (3.13 mg·g^{-1}) mean value on Total phenolic content was 4.97 mg·g^{-1} on dry mass basis (Table 4 and Figure 4.1). The control and P rates above 75 kg·ha^{-1} had similar Total phenolic content.

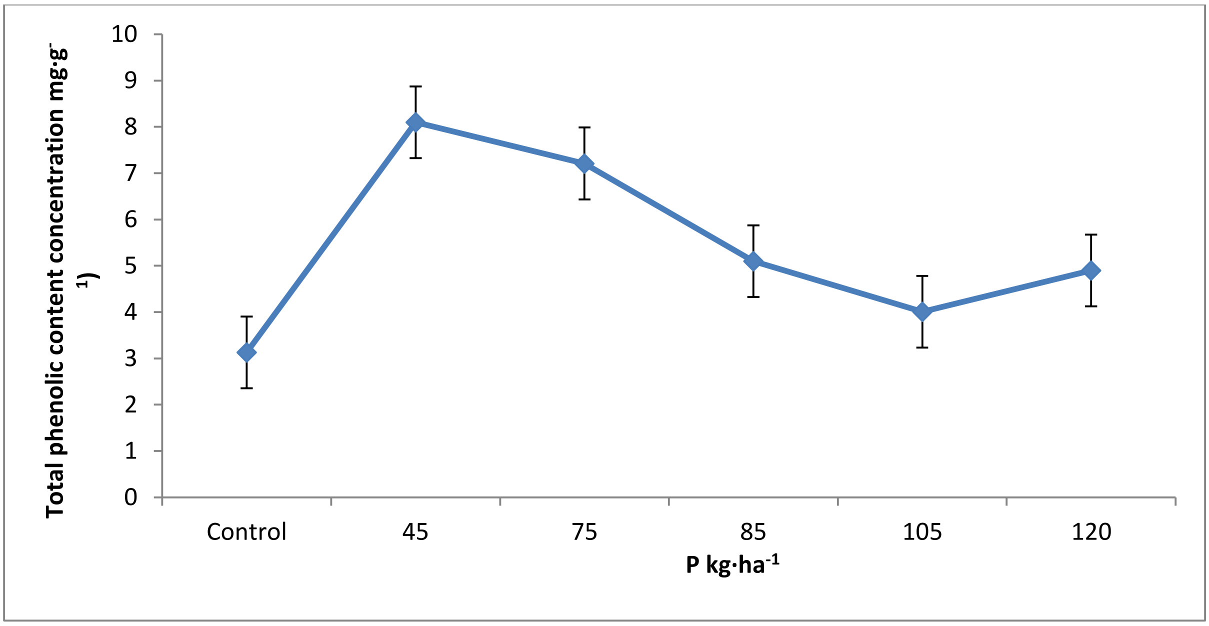

Figure 4.1: Total phenolic content concentrations of baby spinach at different rates of phosphorus application (dry weight basis)

4.3.3 Response of total carotenoid content to phosphorus nutrition

Different levels of phosphorus applied did not have any significant effect on the total carotenoid content in baby spinach leaves with the lowest value of 0.89 mg·g^{-1} and the highest value of 1.60 mg·g^{-1} (Table 2). Thus, there was no significant increase on the concentration of carotenoids regardless of the levels of P applied.

4.3.4 Response of total flavonoid content to phosphorus nutrition

The results showed that the total flavonoid concentration was quadratically increased by phosphorus nutrition. Highest concentrations were observed at 45 kg·ha^{-1} (Table 4 and Figure 4.2). Most of the total flavonoid content response to P occurred between 0 to 45 kg·ha^{-1}. The highest total concentration of total flavonoid content was 9.32 mg·g^{-1}and the lowest mean value was 5.13 mg·g^{-1}. The difference between the highest mean value and the lowest mean value is 4.19 mg·g^{-1} (Table 4 and Figure 4.2). The control and P rates of 105 and 120 kg·ha^{-1} achieved similar levels.

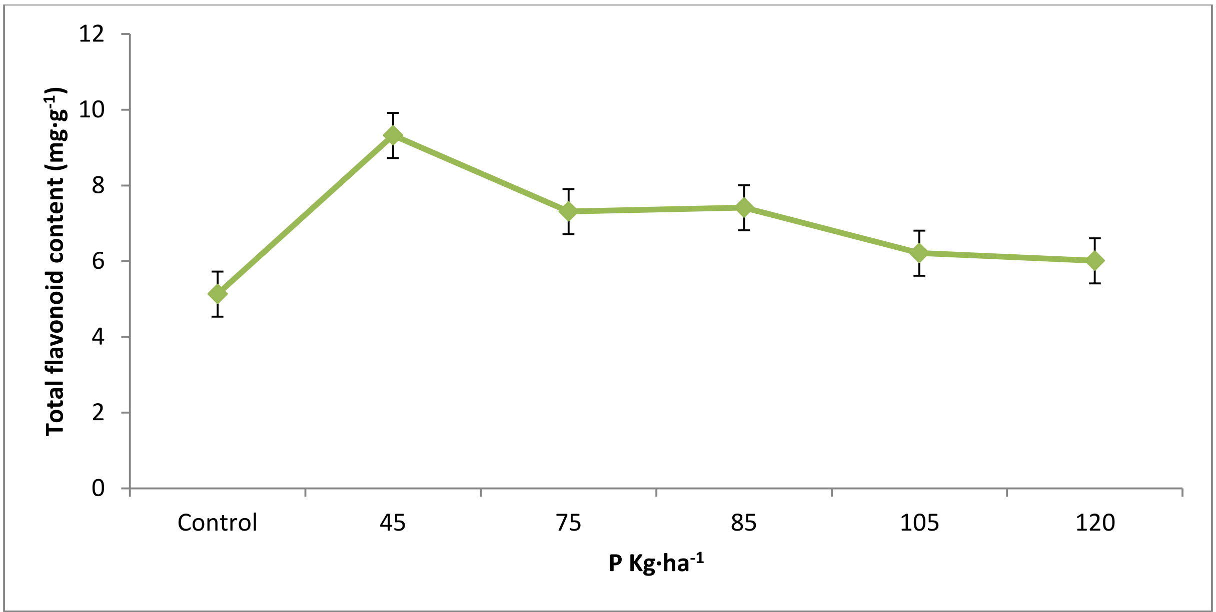

Figure 4.2: Total flavonoid concentrations of baby spinach at different rates of phosphorus application (dry weight basis)

4.3.5 Response of total antioxidant activity of phosphorus nutrition

In the P trial, total antioxidant activity concentration reached their maximum at 45 kg·ha^{-1} with total antioxidant activity concentration of 4.01 mg·g^{-1} (Table 4 and Figure 4.3). Most of total antioxidant activity phenols response to P occurred between 0 to 45 kg·ha^{-1}. The difference between the highest (4.01 mg·g^{-1}) and lowest (3.50 mg·g^{-1}) mean value on Total phenolic content was 0.5 mg·g^{-1} on dry mass basis (Table 4 and Figure 4.3). Application of 45 kg·ha^{-1} achieved significantly higher level of antioxidants than all other treatments.

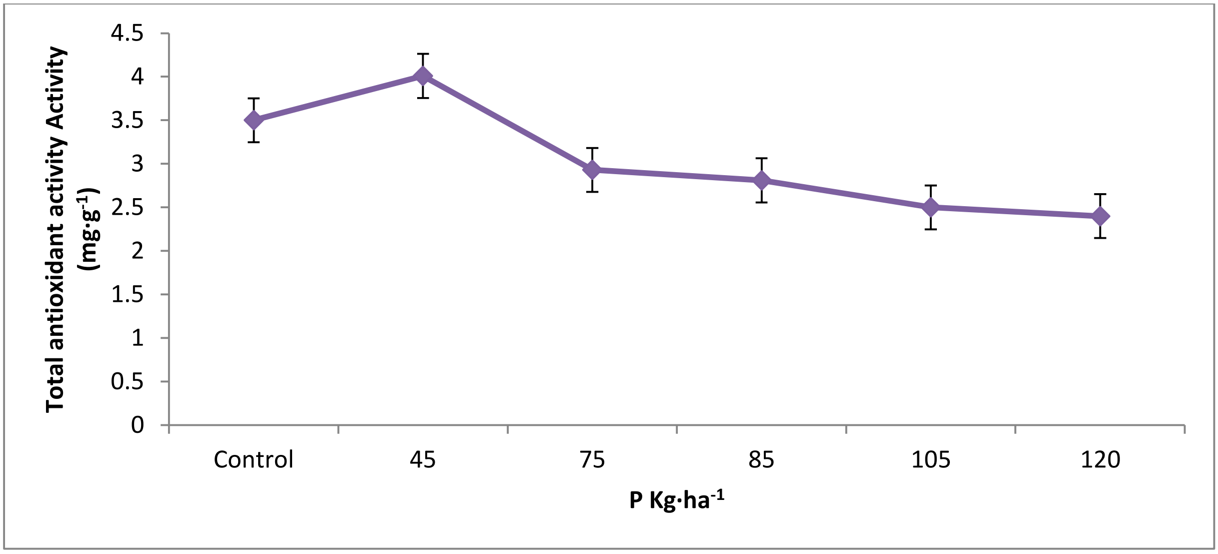

Figure 4.3: Total antioxidant activity concentrations of baby spinach at different rates of phosphorus application (dry weight basis)

4.3.6 Response of Vitamin C to phosphorus nutrition

Results in Table 4 and Figure 4.4 showed a variation in concentrations of vitamin C in baby spinach. There was a quadratically increased in vitamin C due to phosphorus nutrition. Highest concentrations were at 45 kg·ha^{-1}. Vitamin C response to N occurred between 0 to 45 kg·ha^{-1}. The lowest concentration in vitamin C in the leaves of baby spinach was 5.42 mg·g^{-1} and the highest concentrations was 9.9 mg·g^{-1}. The difference between the highest and lowest mean value on total antioxidant activity content was 4.48 mg·g^{-1} on dry mass (Table 4 and Figure 4.4).

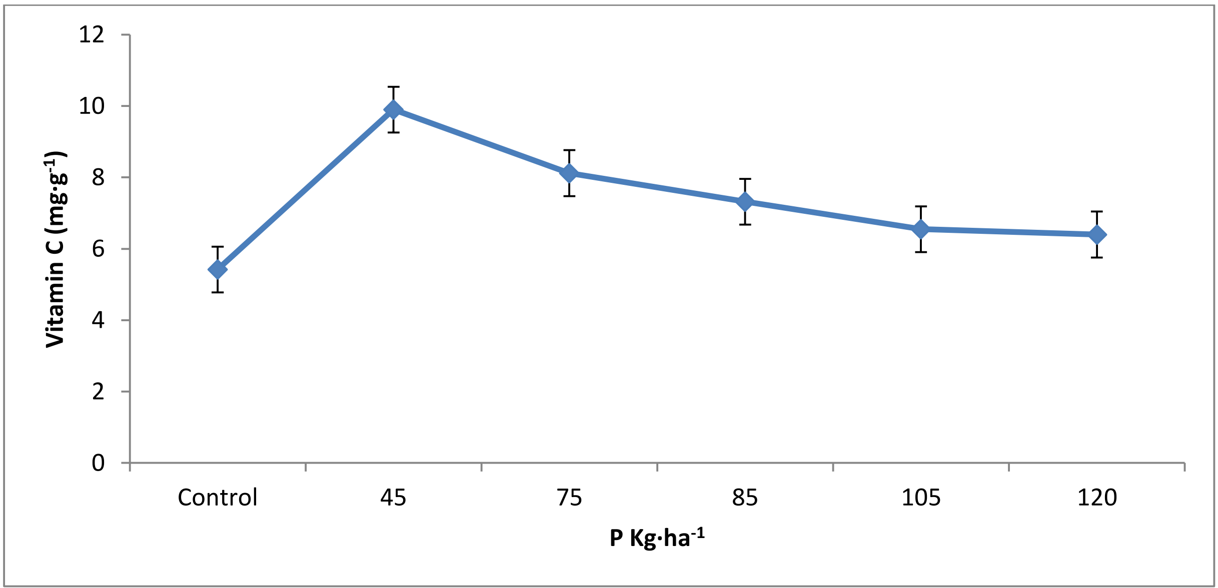

Figure 4.4: Vitamin C concentrations of baby spinach at different rates of phosphorus application (dry weight basis)

4.3.7 Percentage leaf tissue P

Percentage leaf tissue phosphorus was quadratically increased and ranged from 0.526% to 0.968 % (Table 4 and Figure 4.5). Phosphorus applied significantly increased % leaf tissue P of baby spinach compared to control reaching maximum at 45kg·ha^{-1}. The difference between the highest and lowest value was 0.442% (Table 3 and Figure 4.5).

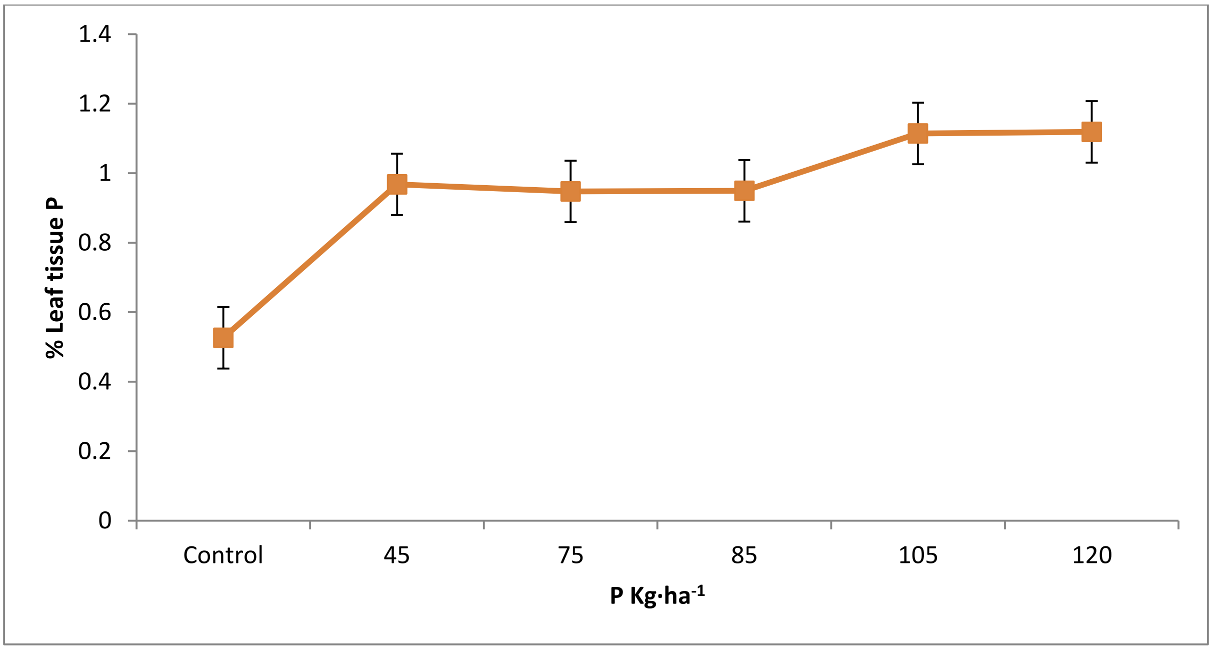

Figure 4.5 Percentage leaf tissue P in baby spinach

4.3.8 Protein %

Protein percentage seemed to have increased at 75kg·ha^{-1} which showed that there was a relationship between the nitrogen applied and protein found in the leaves of baby spinach. Increasing the rates of nitrogen applied to 120kg·ha^{-1} did not exhibit protein content. The difference between the highest value and lowest value was 18.3% (Table 4 and Figure 4.6).

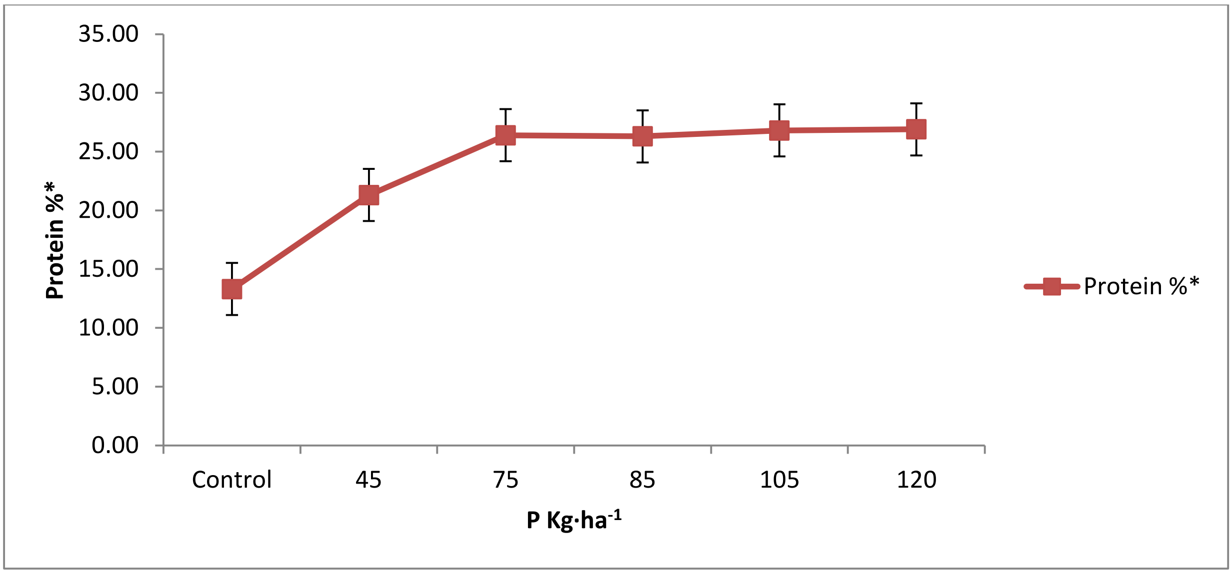

Figure 4.6 Protein % in baby spinach

4.4. Response of chemical composition and minerals to potassium nutrition

Table 5 below indicates the variations on total phenolic content, total antioxidant activity, total carotenoid content and vitamin C, magnesium, iron, zinc and selenium in baby spinach as a result of potassium applied at different rates.

Table 5: Response of chemical concentrations in baby spinach to different levels of potassium (K) nutrition (dry weight basis)

Potassium Applied ($kg \cdot ha^{-1}$)	Magnesium (ppm)	Iron (ppm)	Zinc (ppm)	Selenium (ppm)	Total Phenols ($mg \cdot g^{-1}$)	Total Carotenoids ($mg \cdot g^{-1}$)	Total flavonoid content ($mg \cdot g^{-1}$)	Total Antioxidant Activity ($mg \cdot g^{-1}$)	Vitamin C ($mg \cdot g^{-1}$)	% Leaf tissue K	Protein %*
Control	1.23a	0.81a	1.123a	0.221a	1.01b	0.89a	3.01b	0.49b	3.51b	4.61c	10.8b
60	1.11a	0.11a	1.010a	0.201a	3.13b	1.89a	7.13a	0.50b	4.42b	6.25b	14.3a
85	1.12a	0.81a	1.123a	0.211a	7.10a	1.71a	7.10a	5.01a	7.90a	6.63b	14.4a
106	1.11a	0.21a	1.010a	0.201a	6.21a	1.50a	6.21a	3.13a	6.12a	7.60a	11.1b
127	1.11a	0.91a	1.123a	0.221a	5.10a	1.48a	6.10a	2.01a	5.12a	7.49a	11.4b
148	1.12a	0.82a	1.021a	0.201a	5.01a	1.39a	6010a	2.23a	5.15a	7.40a	11.5b
Significance	Ns	Ns	Ns	Ns	Q	Q	Q	Q	Q	Q	Q

Linear (L) or quadratic (Q) effects significant at P = 0.05 (*), 0.01 (**) or NS

Means with the same letter are not significantly different at 5% level of probability

4.4.1 Response of minerals in baby spinach in potassium nutrition

Results in Table 5 showed that regardless of different levels of potassium applied, there was no significant effect on the minerals magnesium, iron, zinc and selenium concentrations of baby spinach.

4.4.2 Response of total phenolic content to potassium nutrition

The results in Table 5 and Figure 5.1 indicate that the total phenolic content concentration increased quadratically in response to potassium application. Total phenolic content concentration level peaked at 85kg·ha^{-1}. The application of 85kg·ha^{-1} improved the total polyphenol content reaching 7.10 mg·g^{-1} (Table 5 and Figure 5.1). Most of the total phenolic content response to K occurred between 0 to 85 kg·ha^{-1}. The difference between the highest (7.10 mg·g^{-1}) and lowest (1.01 mg·g^{-1}) mean value on Total phenolic content was 6.09 mg·g^{-1} on dry mass basis (Table 5 and Figure 5.1).

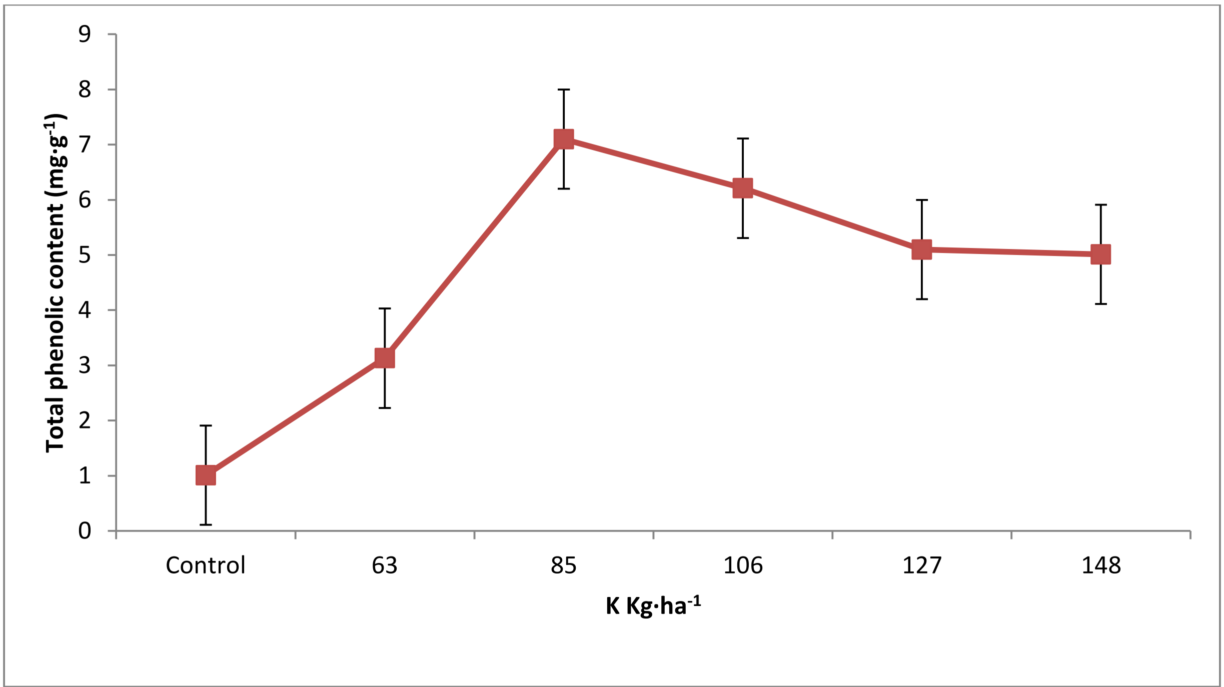

Figure 5.1: Total phenolic content concentrations of baby spinach at different rates of potassium application (dry weight basis)

4.4.3 Response of total carotenoid content to potassium nutrition

Different levels of potassium applied did not have any significant effect on the total carotenoid content in baby spinach leaves. In K trial, there was no significant increase on the concentration of carotenoids regardless of the levels of K applied (Table 5).

4.4.4 Response of total flavonoid content to potassium nutrition

The results showed that the total flavonoid concentration quadratically increased by potassium nutrition. Highest concentrations were observed at 63 kg·ha^{-1} (Table 5 and Figure 5.2). Most of the total flavonoid content response to K occurred between 0 to 85 kg·ha^{-1}. The highest total concentration of total flavonoid content was 7.13 mg·g^{-1}and the lowest mean value was 3.01 mg·g^{-1} (Table 5 and Figure 5.2). The difference between the lowest and the highest mean values was 4.12 mg·g^{-1}.

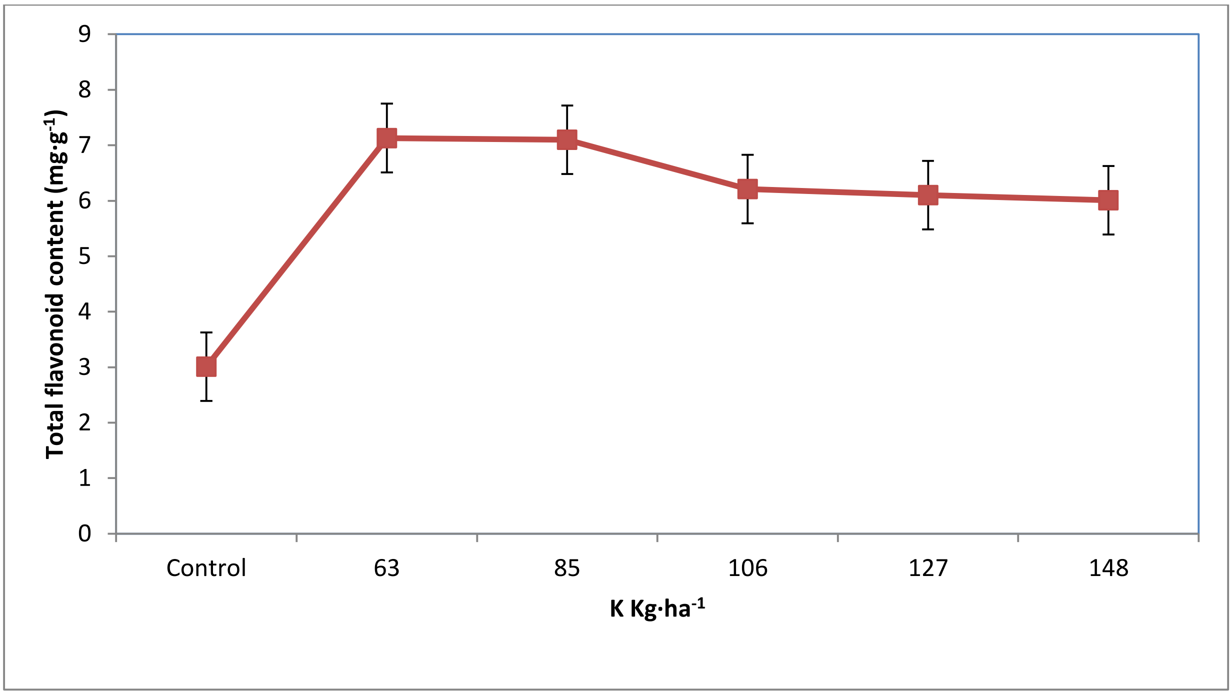

Figure 5.2: Total flavonoid concentrations of baby spinach at different rates of potassium application (dry weight basis)

4.4.5 Response of total antioxidant activity to potassium nutrition

The total antioxidant activity content peaked at 85 kg·ha^{-1} reaching a maximum of 5.01 mg·g^{-1} (Table 5 and Figure 5.3). The highest mean value was 5.01 mg·g^{-1} and the lowest was 0.49 mg·g^{-1}. The difference between the highest and lowest mean value on total antioxidant activity content was 4.52 mg·g^{-1} on dry mass basis (Table 5 and Figure 5.3).

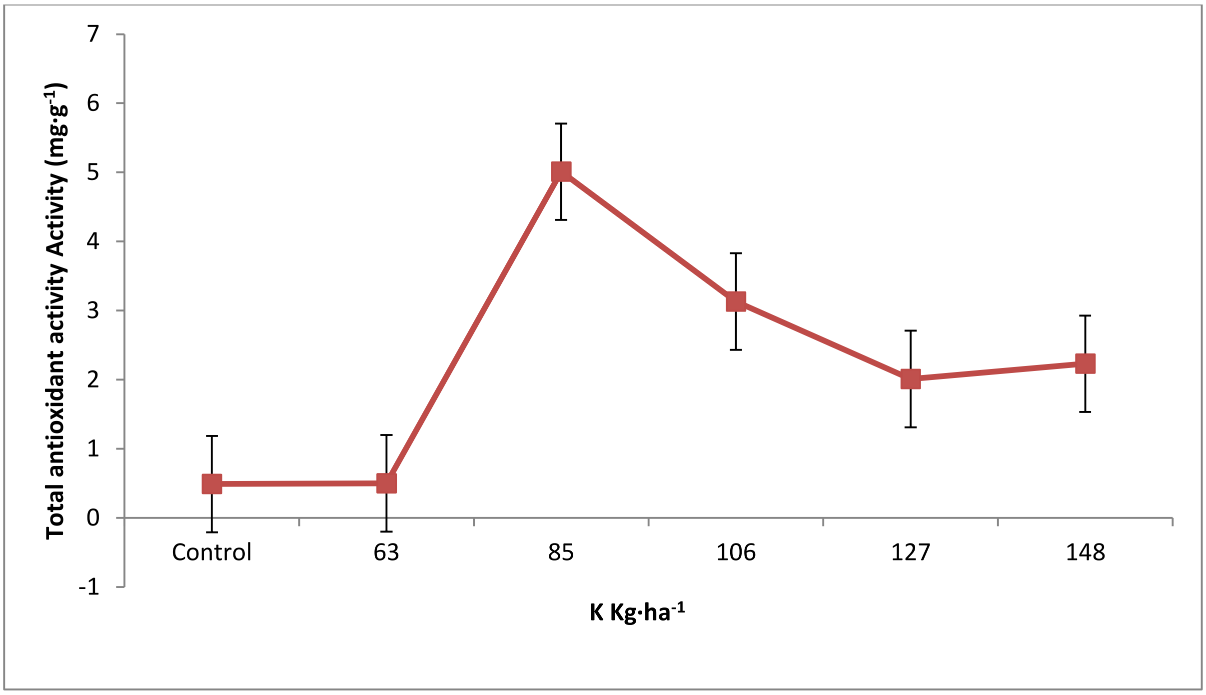

Figure 5.3: Total antioxidant activity concentrations of baby spinach at different rates of potassium application (dry weight basis)

4.4.6 Response of Vitamin C to potassium nutrition

Results in Table 5 indicted that there was a variation in concentrations of vitamin C in baby spinach. Vitamin C was quadratically increased by nutrition. Highest concentrations were at 85 kg·ha^{-1}. Vitamin C response to N occurred between 0 to 85 kg·ha^{-1}. The lowest vitamin C in the leaves concentrations was 3.51 mg·g^{-1} and the highest concentrations was 7.90 mg·g^{-1} (Table 5 and Figure 5.4). The difference between the highest and lowest mean value on total antioxidant activity content was 4.39 mg·g^{-1} on dry mass basis (Table 5 and Figure 5.4).

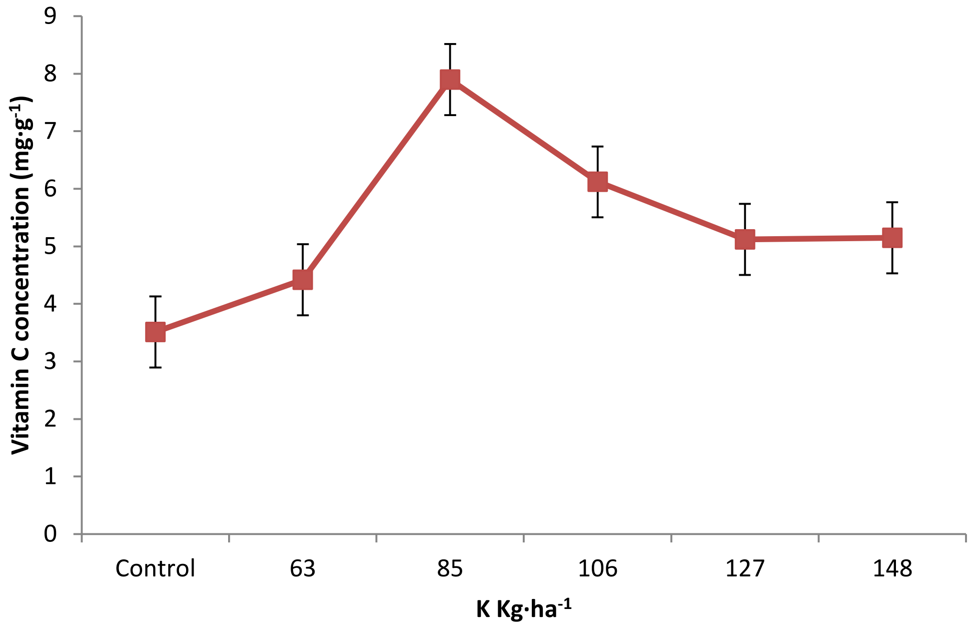

Figure 5.4: Vitamin C concentrations of baby spinach at different rates of potassium application (dry weight basis)

4.4.7 % Leaf tissue K

Potassium applied significantly increased % protein content (value) of baby spinach compared to control reaching maximum at 45kg·ha^{-1}. The difference between the highest and lowest value was 2.88% (Table 5 and Figure 5.5).

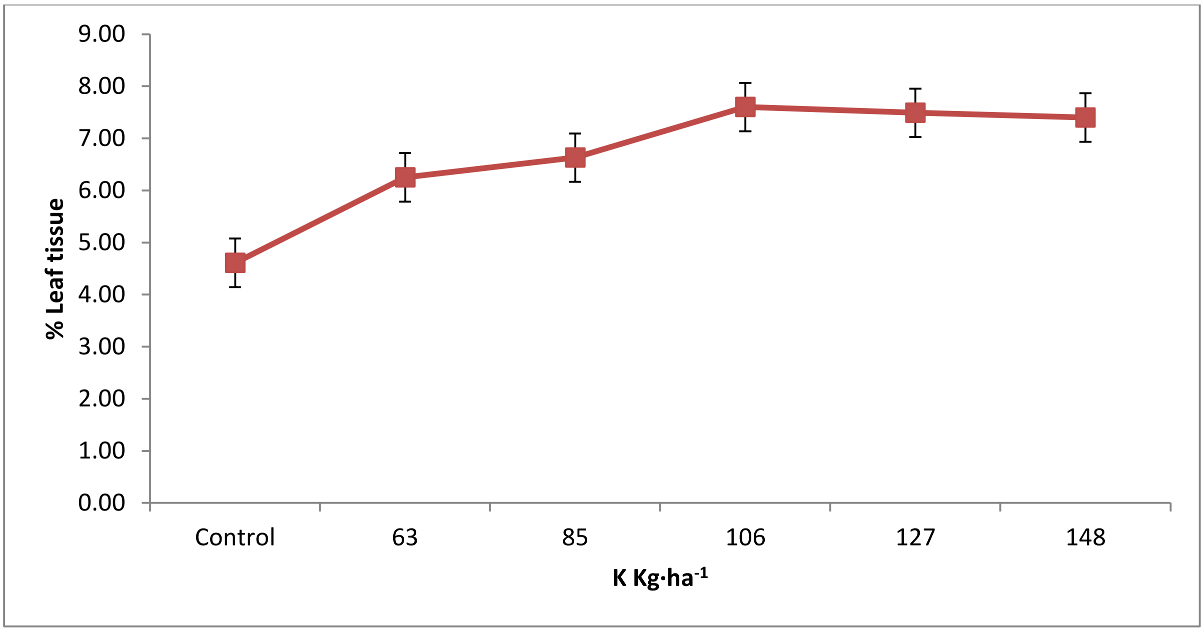

Figure 5.5: % Leaf tissue K in baby spinach

4.4.8 Protein %

Protein percentage seemed to have increased at 63 kg·ha^{-1} which showed that there was a relationship between the nitrogen applied and protein found in the leaves of baby spinach. Increasing the rates of nitrogen applied to 148 kg·ha^{-1} did not exhibit protein content. The difference between the highest value and lowest value was 18.3% (Table 5 and Figure 5.6).

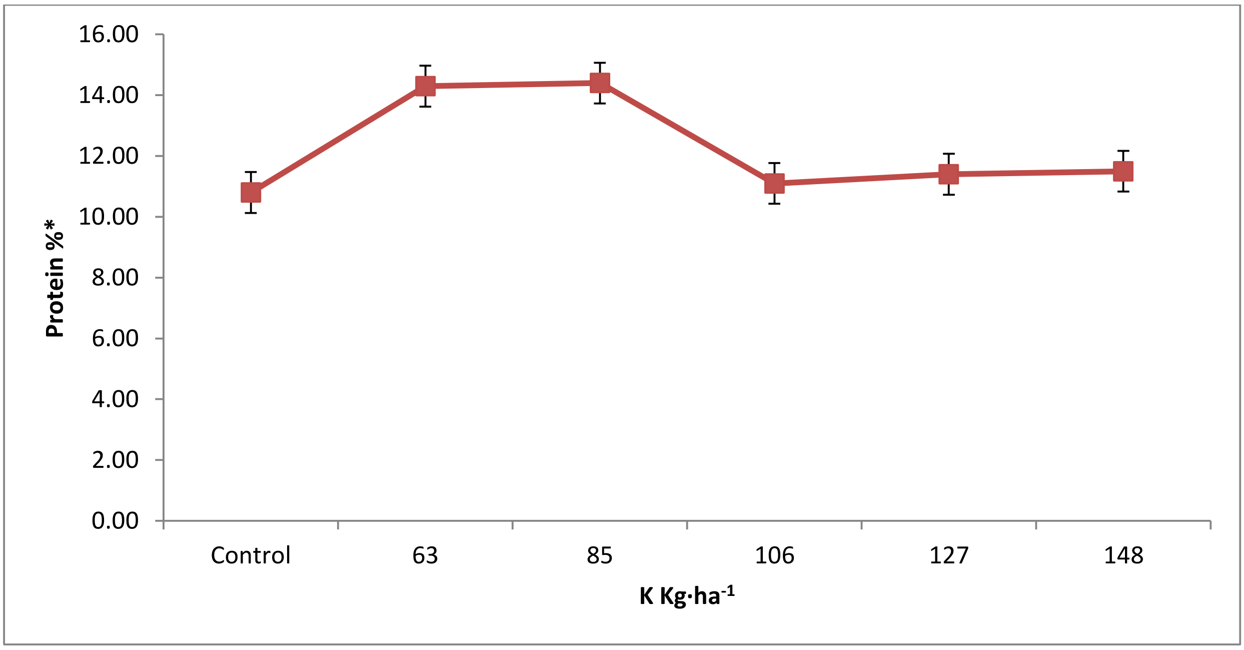

Figure 5.6: Protein%* in baby spinach

4.5 Effect of combinations of different rates of nitrogen, phosphorus and potassium (NPK) fertilizers on chemical concentrations in baby spinach

Table 6 below indicates the variations on total phenolic content, total carotenoid content, total antioxidant activity, total flavonoid content, vitamin C, magnesium, iron, zinc and selenium in baby spinach as a result of combinations of different rates of nitrogen, phosphorus and potassium fertilizers applied.

Table 6: Response of chemical composition in baby spinach to different levels of nitrogen (N), phosphorus (P), and potassium (K) treatment combinations nutrition (dry mass basis)

Nitrogen/ Phosphorus /Potassium Applied ($kg·ha^{-1}$)	Magnesium (ppm)	Iron (ppm)	Zinc (ppm)	Selenium (ppm)	Total Phenols ($mg·g^{-1}$)	Total Carotenoids ($mg·g^{-1}$)	Total flavonoid content ($mg·g^{-1}$)	Total Antioxidant Activity ($mg·g^{-1}$)	Vitamin C ($mg·g^{-1}$)	Protein %*	% N	% P	% K
Control	1.23a	0.81a	1.123a	0.221a	1.01b	0.89b	3.01b	0.49b	3.51a	20.0b	3.21b	0.19a	3.45b
30:30:45	1.11a	0.11a	1.01a	0.201a	5.01a	1.89a	3.01b	4.49a	3.51a	27.8a	4.51a	0.45a	5.90a
45:45:60	1.12a	0.81a	1.123a	0.211a	6.13a	3.89a	7.13a	6.50a	4.42a	25.4a	4.10a	0.49a	5.79a
75:75:90	1.11a	0.21a	1.01a	0.201a	5.10a	1.71a	5.10a	3.01a	3.91a	26.5a	4.30a	0.60a	5.86a
60:60:75	1.11a	0.91a	1.123a	0.221a	4.21a	1.50a	4.21a	2.13b	2.12a	27.1a	4.42a	0.53a	6.13a
75:75:90	1.12a	0.82a	1.021a	0.201a	4.10a	1.48a	3.10a	2.01b	2.10a	27.3a	4.47a	0.50a	6.19a
Significance	Ns	Ns	Ns	Ns	Q	Q	Q	Q	Ns	Q	Q	Ns	Q

Linear (L) or quadratic (Q) effects significant at P = 0.05 (*), 0.01 (**) or NS

Means with the same letter are not significantly different at 5% level of probability

4.5.1 Response of minerals in baby spinach to NPK nutrition

The treatment combinations of NPK rates applied on baby spinach did not show any significance effect on the magnesium, iron, zinc and selenium concentrations (Table 6). Therefore, based on Table 6 there were no significant differences observed on minerals regardless of different treatment combinations of NPK applied (Table 6).

4.5.2 Response of total phenolic content to NPK nutrition

The treatments of N45, P45, K60 $kg{\cdot}ha^{-1}$ improved the concentrations of total phenolic content (Tables 6 and Figure 6.1). This is in agreement with the treatment combination of NPK (45:45:60) which indicated a significantly increased of total phenolic content in baby spinach compared to the control. The mean value of total phenolic content at NPK combination (45:45:60) was 6.13 $mg{\cdot}g^{-1}$ higher than all other treatments (Table 6 and Figure 6.1).

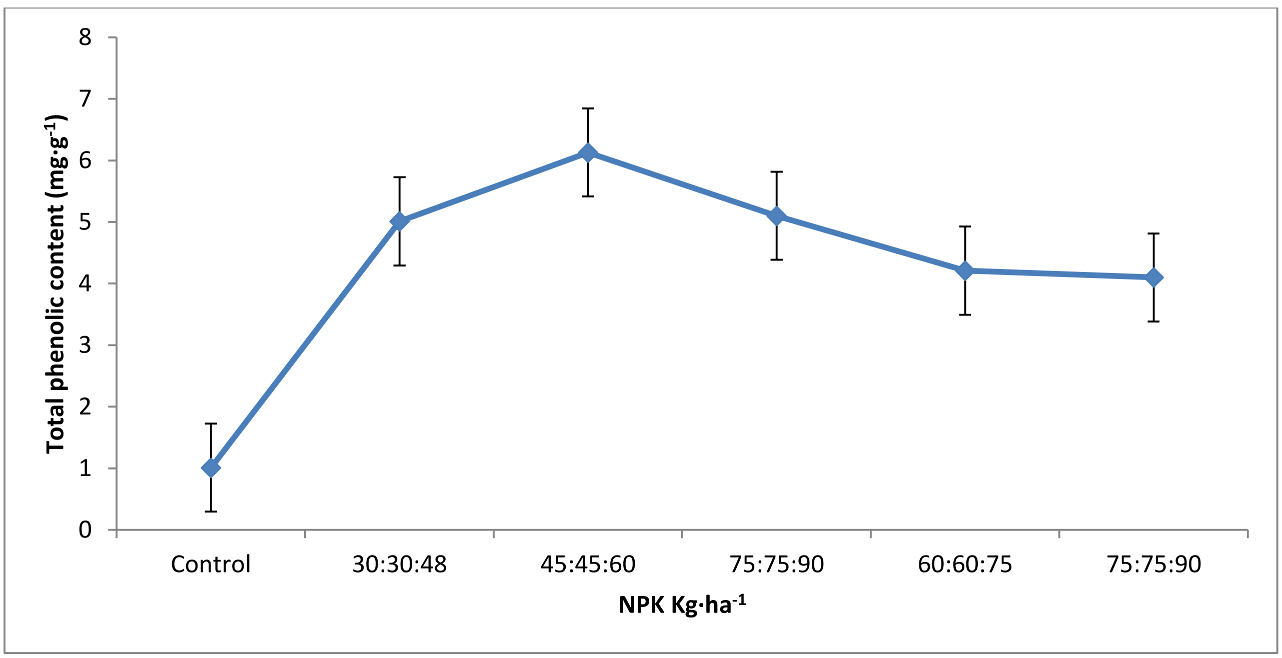

Figure 6.1: Total phenolic content concentrations of baby spinach at different rates of NPK application (dry weight basis)

4.5.3 Response of total carotenoid content to NPK nutrition

Total flavonoid content was not significantly affected by treatment combinations of NPK fertilizers, although the plants under N45, P45, K60 kg·ha^{-1} had the highest total flavonoid content of 3.89 mg·g^{-1} compared to other treatment combinations including the control (Table 6 and Figure 6.2). There was no significant difference in total carotenoid content among other treatment combinations of NPK. The difference between the highest mean value and the lowest mean value was 3 mg·g^{-1}.

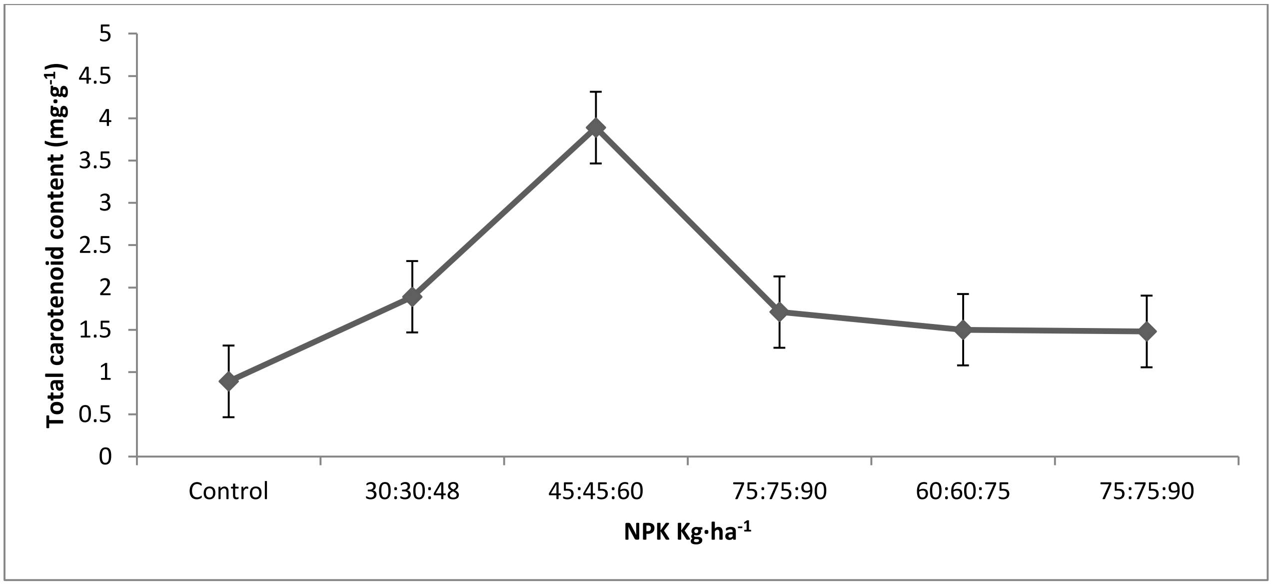

Figure 6.2: Total carotenoid content concentrations of baby spinach at different rates of NPK application (dry weight basis)

4.5.4 Response to total flavonoid content to NPK nutrition

Total flavonoid content was significantly affected by NPK treatment combinations. The plants under N45, P45, K60 kg·ha^{-1} were significantly affected, reaching highest total flavonoid content of 7.13 mg·g^{-1} compared to the control and other treatment combinations (Table 6 and Figure 6.3). There was no significant difference in total flavonoid content among other treatment combinations of NPK and a slight decrease in the total flavonoid content was observed with the maximum treatment combinations.

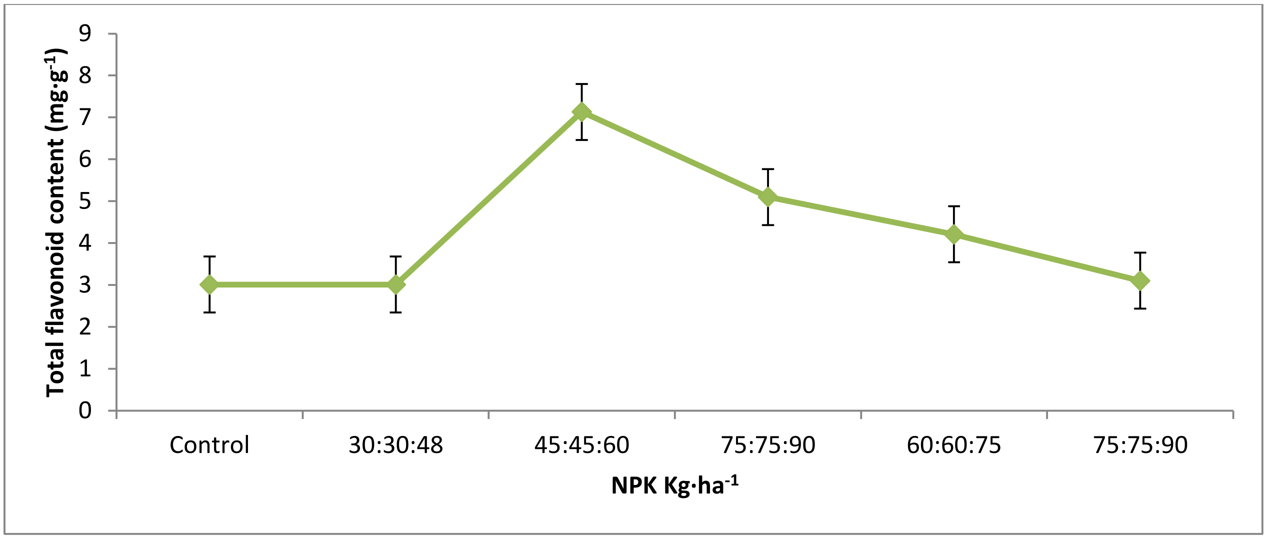

Figure 6.3: Total flavonoid concentrations of baby spinach at different rates of NPK application (dry weight basis)

4.5.5 Response of total antioxidant activity content to NPK

The effect of NPK on the antioxidant activities and components of baby spinach leaves is presented in (Table 6 and 6.4). The plants under N45, P45, K60 kg·ha^{-1} had the highest antioxidant activity of 6.5 mg·g^{-1} compared to other treatment combinations including the control (0 kg·ha^{-1}). The Total phenolic content of plants under N45, P45, K60 kg·ha^{-1} was significantly higher than the plants under other NPK levels (Table 6 and Figure 6.4).

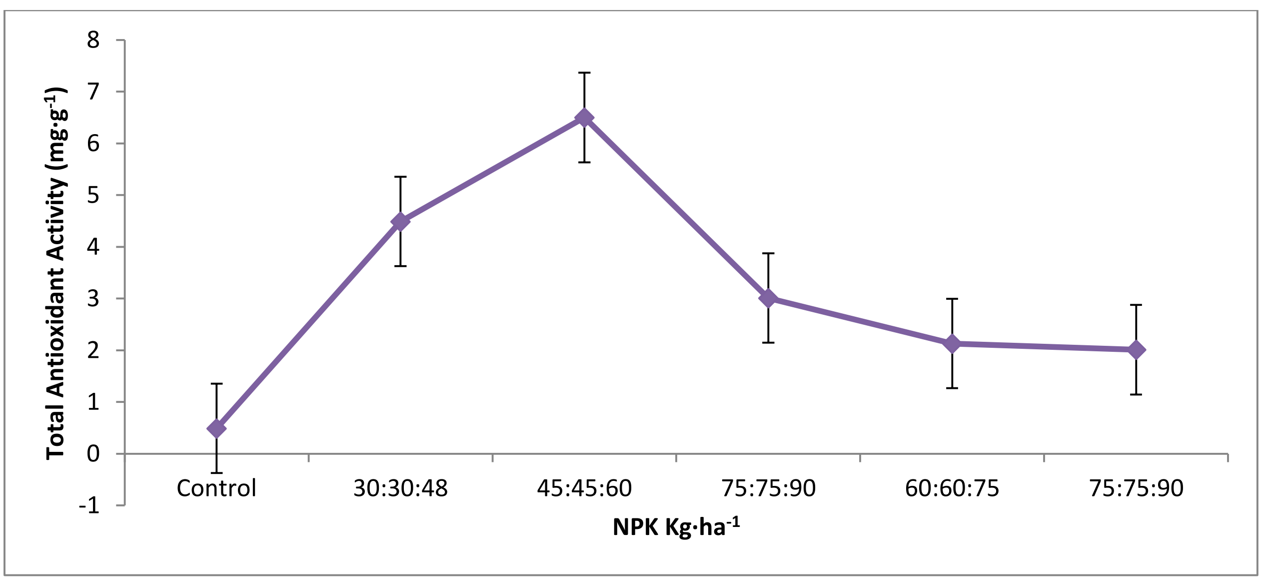

Figure 6.4: Total antioxidant activity concentrations of baby spinach at different rates of NPK application (dry weight basis)

4.5.6 Response of Vitamin C content to NPK nutrition

The results showed that NPK treatment combinations applied on baby spinach did not show any significance influence on the vitamin C content.

4.5.7 Percentage protein content to NPK nutrition

The treatment combinations of N30, P30, K48 (kg·ha^{-1}) increased leaf protein content percentage ranging from 20 to 27.8% (Table 6). Treatment combinations of N30, P30, K48 kg·ha^{-1} significantly improved leaf protein content percentage (Table 6).

4.5.8 Percentage leaf tissue N to NPK nutrition

Percentage leaf tissue potassium was increased and ranged from 3.21 to 4.51% (Table 6). The treatment combinations of N30, P30, K48 (kg·ha^{-1}) improved the concentrations of leaf tissue N (%) (Table 6)

4.5.9 Percentage leaf tissue P to NPK nutrition

All treatment combinations of NPK rates applied on baby spinach did not show any significance effect on percentage leaf tissue (Table 6)

4.5.10 Percentage leaf tissue K to NPK nutrition

Percentage leaf tissue potassium was increased and ranged from 3.45 to 6.19% (Table 6).

4.6 DISCUSSION

4.6.1 Introduction

This section presents the findings of the study compared with related previous studies, looking at the similarities and the contrasts in the findings. The parameters recorded in this study included total phenolic content, total carotenoid content, total antioxidant activity, total flavonoid content, vitamin C, magnesium, iron, zinc and selenium.

Marketable baby spinach production requires suitable levels of nitrogen (N), phosphorus (P), and potassium (K) to provide high-quality postharvest qualities required for longer shelf life and health benefits.

The following findings are discussed in this section: the effect of different rates of N, P and K fertilizers and the NPK treatment combinations on chemical concentrations (total phenolic content, total carotenoid content, total flavonoid content and total antioxidant activity) and minerals (magnesium, iron, zinc and selenium) in baby spinach. Therefore, the section deliberates on the outcomes of the study in an effort to consolidate and recommend the adequate NPK fertilizer rates or levels suitable for South African baby spinach production.

4.6.2 The response of chemical concentrations and minerals to nitrogen nutrition in baby spinach

Table 3 and Figures 3.1 to 3.6 showed the variation in chemical concentrations in the leaves of baby spinach as affected by applied different levels of nitrogen nutrition.

The results showed that the application of nitrogen at different levels did not have any significant effect on magnesium, iron, zinc and selenium concentrations of baby spinach leaves (Table 3). These nutrients are present in smaller quantities in baby spinach, excluding iron which is regarded as one of the major nutrients in baby spinach (Hedges and Lister, 2007). On the contrary to the results of this study, Lefsrud *et al*. (2007) reported magnesium and zinc responded to nitrogen treatments, zinc decreased as nitrogen level increases while magnesium increases in response to increase in nitrogen treatment level.

Other findings by Wang *et al.* (2008) are that nitrogen fertilization increases the ratio of acids and sugars and reduces of calcium, magnesium and soluble sugars. Studies by Chenard *et al.* (2005) reported that leaf iron, manganese and molybdenum decreased with increases in nitrogen in nutrient solutions. Increasing solution nitrogen resulted in quadratic increases in leaf calcium, magnesium, sulfur and boron Chenard *et al.* (2005).

In this study the results showed that Total phenolic content in baby spinach increased quadratically in response to different levels of applied nitrogen reaching a maximum at 45 $kg{\cdot}ha^{-1}$ as indicated in Table 3 and Figure 3.1. Increasing nitrogenous fertilizer rates had negative effect on total phenolic content of baby spinach as total phenolic content were the lowest at 120 $kg{\cdot}ha^{-1}$N. This suggests that total phenolic content of baby spinach deteriorated with increasing nitrogenous fertilizer rates. The similar results were reported by Li *et al.* (2008) that total phenolic content decreased with increasing nitrogen application, the highest content was observed when the level of nitrogen was 100 $kg{\cdot}ha^{-1}$

Clearly there was negative correlation between nitrogen application and Total phenolic content in the baby spinach leaves. The negative correlation between nitrogen application and Total phenolic content could be explained by the protein competition model (PCM) (Jones and Hartley, 1999). The PCM hypothesis makes a conditional prediction that there will be trade-offs between plant growth and Total phenolic content. This elucidates that when biomass increases in response to high nitrogen application, Total phenolic contents will decline because increased protein demand for growth will decrease dividing of carbon skeletons to Total phenolic contents (Jones and Hartley, 1999). Haukioja *et al*. (1998) stated that the contradiction of the results of total phenolic content were largely due to lower leaf nitrogen content, seemingly due to increase in carbohydrate concentration when plants were stressed.

According to Bryant *et al.* (1983; 1987) the carbon/nutrient balance (CNB) hypothesis suggest that growth is limited by deficiencies in carbon or nitrogen while rates of photosynthesis remain unaffected, and the following reduced growth results in the more abundant resource being invested in improved defence (mass-balance based allocation). Other studies showed when there is deficiency in nitrogen or nitrogen applications that significantly depressed yield resulted in the highest content of phenolic compounds in Chinese cabbage (Zhu *et al*., 2009), broccoli heads (Jones *et al*., 2007), lettuce (Coria-Cayupan *et al*., 2009). This suggests that there was a strong trade-off in nutrients directed towards the production of total phenolic content.

The results showed that different nitrogen levels applied did not have any significant effect on the total carotenoid content of baby spinach leaves (Table 3). This suggests that total carotenoid content did not respond to any level of nitrogen applied. These results are contrary to the results reported in other studies, where the mid-range applications of nitrogen were most effective in lettuce at 0–1.6g·100g (Coria-Cayupan *et al*., 2009). Lefsrud *et al.* (2007) reported that lutein accumulations, expressed on a fresh weight basis, responded quadratically to increasing nitrogen treatments. The results showed that increasing nitrogen application increased the content of beta-carotene, but when the level of nitrogen was higher than 200 kg·ha^{-1}, their contents remained nearly constant (Li *et al.,* 2008). King *et al.* (2008) reported the effects of nitrogen and phosphorus fertilization on total carotenoid content in spinach and established that increases in nitrogen fertilization from 0 to 300 kg·ha^{-1} had slight effect on total carotenoid content.

The effects of different nitrogen application rates on total flavonoid content are summarized in Table 3 and Figure 3.2, showing the range of applied nitrogen and the rate at which optimal total flavonoid content was reached at 45kg·ha^{-1}. In this study, it is notable that the lowest nitrogen application rate resulted in highest flavonoid content in baby spinach leaves.

The results of the study suggested that the application of 45kg·ha^{-1} improved the total antioxidant activity content reaching 3.01 mg·g^{-1} (Table 3 and Figure 3.3). The total antioxidant activity content of plants under 45 kg·ha^{-1} was significantly higher compared to the control. It was further observed on the results that excessive application of nitrogen had a negative effect on total antioxidant activity in baby spinach. This was also reported by Mozafar (1993) that higher levels of nitrogen increases concentration of nitrate (NO_3^-) and simultaneously decreases the concentration of total antioxidant activity in vegetables. Similar results were reported by Briemer (1982) that increased levels of nitrogen increased concentration of nitrate while decreased the concentration of total antioxidant activity in vegetables. Kopsell *et al*. (2007) reported that nitrogen fertilization has effect on quality parameters in leafy vegetables.

Nitrate percentage in the baby spinach leaves increased with increasing nitrogen application reaching a maximum of 1.40% at 120 kg·ha^{-1} (Table 3). The results of statistical analysis indicated that the effects of nitrogen levels on nitrate accumulation were significant (Table 3). These results are in agreement with Mozafar (1993) who reported that the over application of nitrogen fertilizers increases the concentration of nitrate. However, the nitrate content in this study was acceptable as per World Health Organization (WHO) standards. The WHO calculated the daily intakes of ≤500 mg of sodium nitrate per kg body weight were harmless to rats and dogs. This figure was divided by 100 to yield an Acceptable Daily Intake (ADI) for humans of 5 mg sodium nitrate or 3.7 mg nitrate per kg body weight, which equals 222 mg for a 60 kg adult (Katan, 2009).

Many vegetables and fruits contain 200–2500 mg of nitrate per kilogram (Van Duijvenboden and Matthijsen, 1989). The nitrate content of vegetables can be affected by factors such as processing of the food, the use of fertilizers and growing conditions, especially the soil temperature and (day) light intensity (Gangolli *et al*., 1994; FAO/WHO, 1995). Vegetables such as beetroot, lettuce, radish and spinach often contain nitrate concentrations above 2500 mg·kg^{-1} especially when they are cultivated in greenhouses (FAO/WHO, 1995).

In addition, another study by Szwonek (1986) reported that the concentration of nitrate nitrogen in lettuce and spinach leaves is reliant on the rate of applied nitrogen fertilizer. For example, a significant increase of nitrates in lettuce and spinach was observed when N fertilizer application was increased from 260 to 280 kg·ha^{-1} (Szwonek, 1986). The results of this study are consistent with those reported by Hammad *et al*. (2007); Gulser

(2005) and Briemer (1982) who also reported that the application of high nitrogen levels increases the accumulation of nitrate in vegetables. Sorensen (1999) reported that increased application of nitrogen fertilizer increased the content of nitrates and carotenes.

The results of this study clearly indicate that nitrogen applied had an effect on the vitamin C concentrations in the baby spinach leaves. Vitamin C was quadratically increased by nitrogen application reaching maximum at 45 kg·ha^{-1} (Table 3 and Figure 3.4) but high rate of nitrogen application seem to decrease the concentrations of vitamin C in the baby spinach leaves. These results are in agreement with Mozafar (1993) who reported that the increased levels of nitrogen application decrease the concentration of vitamin C in many fruits and vegetables. However, other reports by Kansal *et al*. (1981); Dzida and Pitura (2008) showed that vitamin C and sugar content in spinach and Swiss chard increases under the application of high rates of nitrogen fertilizers.

Lisiewska and Kmiecik (1996) stated that increasing the quantity of nitrogen nutrition from 80 to 120 kg·ha^{-1} decreased the vitamin C content by 7% in cauliflower. Likewise, Weston and Barth (1997) reported that nitrogen fertilization increases β-carotene in vegetables. Although vitamin C concentration has been found to be positively associated with the nitrogen supply in butterhead lettuce (Muller and Hippe, 1987), it is inversely associated with the nitrogen supply in white cabbage (Sorensen, 1984; Freyman *et al*., 1991) and crisp head lettuce (Sorensen *et al*., 1994). According to Wang *et al.* (2008), nitrogen fertilization increases the ratio of acids and sugars and reduces of vitamin C, calcium, magnesium and soluble sugars. Increased application of nitrogen reduced the content of dry matter, potassium, sucrose, vitamin C and fiber in leafy vegetables, but increased the content of nitrates and carotenes (Sorensen, 1999).

Although according to Uher *et al.* (2013), nitrogen fertilization positively influenced the bioactive compounds and minerals (vitamin C, E1 and ß-carotene) content in cauliflower edible heads. Increased fertilization doses of nitrogen significantly increased vitamin C and ß-carotene content in the cauliflower (Uher *et al*., 2013). This validates some recorded findings on fruits and vegetables by Skwarylo-Bednarz and Krzepilko (2008) who reported that the use of mineral fertilizer, mainly nitrogen increases the vitamin C content. Bergquist (2006) reported that an increase in vitamin C concentration often goes hand in hand with a decrease in carotenoid or flavonoid concentration, and vice versa.

Stamp (2003) suggested a model for explaining the relationship between nutrient availability in the soil and the growth and differentiation in plants called the growth-differentiation balance (GBD) hypothesis. The GBD hypothesis proposes that the plant allocates nutritive resources to growth if available in ample supply, but if resources are constrained the plant diverts more resources to the synthesis of secondary metabolites associated with differentiation. This suggests that if nitrogen is available, the plant diverts more input into nitrogen-containing metabolites, such as protein, vitamin A and nitrate, while more C containing compounds are produced when nitrogen is more restricted, such as vitamin C and many of the secondary metabolites. Stamp (2003) demonstrated further that when resource availability is critically low both growth and secondary metabolite synthesis is hampered.

Nitrogen application significantly increase percent leaf tissue nitrogen of baby spinach compared to control, reaching maximum at 75kg·ha^{-1}. The highest percentage total leaf nitrogen was observed at 120 kg·ha^{-1}, causing a linear response with high rates of nitrogen applied (Table 3). Protein percentage seemed to have increased at 45kg·ha^{-1} which indicated that there is a correlation between the nitrogen applied and protein found in the leaves of baby spinach. Increasing the rates of nitrogen applied to 120kg·ha^{-1} did not exhibit increase in protein content. Higher nitrogen fertilizer application levels increased the tissue nitrogen concentrations. These results are in agreement with Hoque *et al.* (2010) who reported that a higher nitrogen fertilizer application increases the tissue nitrogen concentrations in lettuce.

4.6.3 Response of chemical composition and minerals to phosphorus nutrition in baby spinach

Table 4 and Figures 4.1 to 4.6 showed the variation in chemical concentrations in the leaves of baby spinach as affected by applied different levels of phosphorus nutrition.

Limited studies are available on the effect of P nutrients on baby spinach postharvest quality specifically chemical composition of baby spinach. Plants use phosphorus fertilizer for seed germination and root development and growth (Colomb *et al*., 2000). Despite increasing the yield, phosphorus improves the quality of vegetables in several aspects, such as the chemical composition, the marketability and the storability of the crop (King *et al*., 2008).

In the P trial, it was observed that different levels of phosphorus applied did not have any significant effect on the total carotenoid content in baby spinach leaves (Table 4). Thus, there was no significant increase on the concentration of total carotenoid content regardless of the levels of P applied. Contrary to the findings of this study, King *et al*. (2008) reported the effects of phosphorus fertilization on total carotenoid content in spinach, and reported that the highest carotenoid contents were obtained with maximized phosphorous application reaching 50 $kg \cdot ha^{-1}$.

Based on the results of the current study it can be concluded that the increment of P fertilizer in baby spinach had a negative effect on the concentrations of bioactive compounds such as total phenolic content, total flavonoid content, total antioxidant activity and vitamin C. These results are consistent with those of earlier studies in which the secondary metabolite concentrations improved in P-deficient plants (John *et al*., 2009; Lei *et al*., 2011). To the contrary, other reports suggest that in crops such as lettuce, the results show a distinct yield and quality response to P fertilizer under most conditions (Alt, 1987; Johnstone *et al*., 2005; Sanchez *et al*., 1988). Similarly, according to Soundy and Smith (1992), a significant positive linear correlation between soil P and lettuce head tissue P was observed. This may be supported by the report of Cleaver and Greenwood (1975) who reported that lettuce had higher P fertilizer requirements than most other vegetables across a range of soils. It is evident that there is not much information on the effect of P fertilizer on the bioactive compounds on green leafy vegetables specifically baby spinach.

4.6.4 Response of chemical composition and minerals to potassium nutrition in baby spinach

Table 5 and Figures 5.1 to 5.6 showed the variation in chemical concentrations in the leaves of baby spinach as affected by applied different levels of potassium nutrition.

Potassium is known as one of the standard plant nutrients vital for yield production and quality determination of crop; and promotes flowering, fruiting and good colour in plants (Bergquist, 2006). It has been reported that among plant nutrients potassium has the strongest impact on crop quality parameters that determine consumer preference (Jifon and Lester, 2009). Potassium had been reported as an important macronutrient for plants and is needed for vital functions in metabolism, growth, and stress adaptation (Ismail *et al*., 1994; Rosa *et al*., 2001). Studies report different responses of vegetable crops to potassium nutrition. According to Ismail *et al*. (1994), the functions of potassium in solute carriage, protein synthesis, and enzyme activation indicate a close relationship between potassium and metabolism.

As this study was conducted, it was established that there is a shortage of information on the influence of potassium fertilizer on the concentrations of bioactive compounds in green leafy vegetable specifically baby spinach.

The results of this study showed that potassium had influence on total phenolic content, total flavonoid content, total antioxidant activity and vitamin C of baby spinach except on magnesium, iron, zinc, selenium and total carotenoid content. This concurs with the report that potassium is an important macronutrient for plants and is required for vital functions in metabolism, growth, and stress adaptation (Ismail *et al*., 1994; Rosa *et al*., 2001). The results were in agreement with the report that potassium deficiency affects the metabolite concentrations in crops, with negative significances for nutritional quality and mechanical stability (Patrick *et al*., 2009).

4.6.5 Response of chemical composition and minerals to nitrogen, phosphorus and potassium nutrition in baby spinach

Table 6 and Figures 6.1 to 6.4 showed the variation in chemical concentrations in the leaves of baby spinach as affected by applied different levels of potassium nutrition. The parameters recorded in this study included total phenolic content, total carotenoid content, total antioxidant activity, total flavonoid content, vitamin C, magnesium, iron, zinc and selenium.

The applications of combined nitrogen, phosphorus and potassium (NPK) have been a standard agronomic practice (Keen and Zidenberg-Cherr, 2000). Optimal fertilizer management and efficient use of NPK are necessary to improve yield and quality and to reduce production costs (Fageria, 2009). As this study was conducted, it was established that there is a lack of published reports on the influence of treatment combinations of NPK fertilizer on the concentrations of bioactive compounds in green leafy vegetable specifically baby spinach.

Some studies on related crops such as lettuce has been done by other researchers suggest that N availability in the soil can affect lettuce quality (Tittonell *et al*., 2001) but there is no evidence about the effect of different levels of N, P, and K on the postharvest quality of lettuce. Other studies reported that there may be some yield and quality benefit to N and P fertilizer application because of their role in moderating disease resistance and because of the metabolic functions of K (Marschner, 1995).

From this study it can be concluded that regardless of different levels of NPK nutrition, there is no significant effect on magnesium, iron, zinc and selenium concentrations. However, the concentrations of other parameters such as total phenolic content, total carotenoid content, total flavonoid content, antioxidant activity and vitamin C in baby spinach seem to be influenced by the application of NPK.

The optimum NPK level in this study was N45:P45:K60 $kg \cdot ha^{-1}$ where the total phenolic content, total carotenoid content, total antioxidant activity, total flavonoid content and vitamin C concentrations in baby spinach leaves were higher than all other levels. At N75:P75:K90 $kg \cdot ha^{-1}$ the law of diminishing returns was evident. At this level all concentrations in bioactive compounds decreased due to different levels of NPK applied in the baby spinach leaves.

At N45:P45:K60 $kg \cdot ha^{-1}$ levels of NPK, total phenolic content and antioxidants concentrations of baby spinach leaves were highest (Tables 6). Further increase in the application of NPK resulted in over fertilization and subsequent effect of decline in the antioxidant activity and phenols and antioxidant activity constituents. This is in agreement with the study conducted in China by Juan *et al*. (2008) who reported the effects of N and Sulphur (S) on total phenolics and antioxidant activity of leaf mustard. The authors reported that increasing N supply considerably decreased total phenolic concentrations. Similar findings were reported in addition to the type of soil in accumulation of nutrients by cultivated plants (Kader, 2002; Kader, 1988). The effect of NPK on the antioxidant activities and components of baby spinach leaves were reported in Table 6 and 6.4. The plants under N45, P45, K60 $kg \cdot ha^{-1}$ had the highest antioxidant activity of 6.5 $mg \cdot g^{-1}$ compared to other treatment combinations including the control. These results concur with the results reported by Jeppsson (2000); Kaur and Kapoor (2001); Kemal *et al*. (2008) that mineral fertilization influences antioxidant composition in some fruit and vegetables.

Thus, resulted in the biosynthesis of carbon-based secondary metabolites, such as flavonoids, phenolic acids, and tannins, known as total polyphenols, which are antioxidant in nature (Haukioja *et al*., 1998). Nutrient-deficient plants often have lower growth rates and higher concentrations of carbon-based (non-nitrogen-containing) secondary compounds (CBSCs) than do plants with access to ample nutrients (Bryant *et al*., 1983; Coley *et al*., 1985). This negative correlation between concentrations of CBSCs and plant growth rate, or levels of nutrients in plant tissues, is assumed to suggest or indicate a trade-off between plant growth and the production of defensive compounds (Bryant *et al*., 1987).

The results showed that NPK treatment combinations applied on baby spinach did not show any significance effect on the vitamin C concentration. These findings are in contrary with the report by Somers and Beeson (1948); Lee (1974); Harris (1975); Mozafar (1994); Weston and Barth (1997) who reported that pre-harvest factors such as cultural practices including mineral nutrition are responsible for the wide variation in vitamin C content of fruits and vegetables at harvest.

All treatment combinations of NPK rates applied on baby spinach did not show any significance effect on leaf tissue P percentage (Table 6). Increasing NPK fertilizer application rate had no significant effect on tissue P but had significant effect on N and K of baby spinach. These results concur with the results reported by Hoque *et al*. (2010) that the increasing NPK did not have any significant effect on tissue P or K of lettuce.

4.7 SUMMARY OF RESEARCH FINDINGS

This study investigated the response of chemical composition to NPK fertilization in baby spinach. The following results were observed:

- In N, P and K trials, the application of different levels of fertilizers had significant effect on total phenolic content, total flavonoid content, total antioxidant activity and vitamin C. However magnesium, iron, zinc, selenium and total carotenoid content did not exhibit any significant response to different levels of N, P and K fertilizers applied.
- In NPK treatment combinations, total phenolic content, total carotenoid content, total flavonoid content and total antioxidant activity responded to different levels of NPK treatment combinations. On the other hand different levels of NPK treatment combinations had no significant effect on magnesium, iron, zinc, selenium and vitamin C.

Fertilizer management remains as one of the essential pre-harvest factors in the improvement of health promoting substances in baby spinach.

CHAPTER 5

5.1 CONCLUSION AND RECOMENDATIONS

Besides the maximization of yield in vegetable production research, the main output is to produce healthy vegetables. The question would be what are healthy vegetables? Healthy vegetables referring to vegetables with optimal level of health promoting compounds such as total phenolic content, total carotenoid content, total flavonoid content, total antioxidant activity, vitamins etc. Minerals and bioactive compounds concentration in vegetables can be improved to some limits by best agricultural practices.

Based on the findings of this study, recommended rate of fertilizers in which baby spinach performs best regarding total phenolic content, total antioxidant activity, total flavonoid content and vitamin C is 45 kg·ha^{-1} N and P. The recommended rate for potassium is 85 kg·ha^{-1} where total phenolic content, total antioxidant activity, total flavonoid content and vitamin C concentrations are improved. Where treatment combination of NPK fertilizers was applied, the combination of 45:45:60 kg·ha^{-1} is recommended. The effects of the applications of N, P and K fertilizers at different levels on bioactive compounds were shown to vary greatly. These results have important implications in the recommended amounts of N, P and K fertilizers applied for the cultivation of baby spinach.

Fertilization can decrease or increase the level of concentrations on bioactive compounds depending on the applied level. Adequate levels are recommended in order for these compounds not to be affected negatively but positively in order to achieve the maximum concentrations in bioactive compounds of baby spinach. Therefore, good agricultural practices such as the application of adequate levels of NPK fertilizers are crucial in promoting levels of bioactive compounds in baby spinach.

Finally, it was concluded that fertilizer application had influence on bioactive compounds of baby spinach and adequate levels are critical in improving quality in baby spinach in terms of health related bioactive substances. If adequate levels are applied, bioactive compounds in baby spinach can be improved but excessive application of fertilizer has detrimental effect on the bioactive compounds of baby spinach based on the results of the study.

5.2 FUTURE PROSPECTS

Other factors may also affect bioactive compounds in baby spinach, for an example the method of fertilizer application and timing of application. Therefore, time of application of nitrogen, phosphorus and potassium nutrition and method of application; and their influence on chemical composition in baby spinach are factors to be investigated in future. Another aspect of interest for future studies is to investigate the response of growth (biomass) and development to nitrogen, phosphorus and potassium nutrition in the future. There has been no response reported of magnesium, zinc, iron and selenium on baby spinach to NPK nutrition, suggesting that this is a subject that still has to be investigated in future. The study results are only tentative as they are not analyzed against the soil fertility status of trial site. In future studies there is a need for i) pre-soil analysis, and ii) inclusion of more cultivars of baby spinach.

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The Chemical Composition of Baby Spinach (Spinacia oleracea L.) as Affected by Nitrogen, Phosphorus & Potassium nutrition

Bongekile Octavia Zikalala 3Submitted in accordance with the requirements for the degree of Master of Science in the subject

AGRICULTURE at the UNIVERSITY OF SOUTH AFRICA SUPERVISOR: PROF FHATUWANI NIXWEL MUDAU NOVEMBER 2014 TABLE OF

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_______________________ ___________________ SIGNATURE DATE (MS BO ZIKALALA) ACKNOWLEDGEMENTS 44I would like to thank God Almighty for blessing me with this degree, without Him I wouldn’t be able to achieve so much. He is indeed a God of miracles and possibilities. It

was a tough road and a steep journey, at times I wanted to give up and take another research project but He was always there with me, encouraging me and giving me hope that what seem to be impossible to men it is possible to Him. Apart from my efforts, the 37success of any project depends largely on the encouragement and guidelines of many others. I take this opportunity to express my gratitude to the people who have been instrumental in the successful completion of this project. I am indebted to the following people and their organisations for the positive role they played during the course of this research project: ? I am grateful to Professor Fhatuwani Nixwel Mudau, my research supervisor, for his excellent guidance, commentary and constructive engagement during the course of the study. 65I would like to express my gratitude to him for the useful comments, remarks and engagement through the learning process 73of this master thesis. Furthermore, I would like to thank him for introducing me to this interesting and thought-provoking piece of work. Honestly, 63I can't thank him enough for his remarkable support and assistance. ? Dr Hintsa Arayi is thanked for sharing his knowledge and expertise which enabled me to develop an understanding of the subject. His guidance, supervision, and fundamental contribution, which made him a pillar of this study and so to this thesis. His participation with his creativity has generated and nourished my intellectual maturity that I will benefit from, for a long time to come. Without his inspiration and leadership this project would not have materialized. I would like to thank him whole-heartedly. ? Mr Meshack Mofokeng, a Researcher at the Agricultural Research Council (ARC), is thanked for assistance with respect to the fertilizer calculations and guidance in trial layout. ? Mr Thabo Nkadimeng & Ms Lebogang Phala who are research assistants at Agricultural Research Council (ARC) for being instrumental in providing and preparing all the required apparatus and providing guidance in terms of their usage. ? I offer my respects and dedications to the farm labourers at Agricultural Research Council (ARC, Mr Frankie, Mr Samuel & Mr Obed, You are 53important to the successful realization of this thesis. Thank you so much for your commitment and great help. ? ? My fellow brothers and sisters at Atteridgeville West Church of Christ (AWCOC) for your prayers and support throughout the course of this work. How can I forget my in-laws for their encouraging words and support throughout the project especially Ms Khanyi Ndlela for constantly reminding me the significance and the meaning of this work. ? Where would I be without my family? I have no words to explain my sincere appreciation, I feel so blessed to be part of such a great family. A special thanks to my family. 58Words fail me to express my gratitude to my husband Mr Nhlanhla Zikalala whose devotion, 83love and persistent confidence in me, has made me to be strong and encouraged to pursue my dreams with confidence. I thank him for being unselfish by allowing me to follow my dreams and goals. ? A big thank you to my kids, Amahle, Namandla & Zamakhuze Zikalala for all of the sacrifices that you've made on my behalf. I know there were times where I didn't spend enough time with you as required but you never complained. ? I would like to thank my late parents (Mr & Ms Nene) who taught me to be strong, humble and continue to breathe even when my head is under water. I am a person that I am today because of their guidance and good moral lessons that they imparted in me. I didn't spend much time with them but I thank God for them because they were good parents. ? 41Finally, I would like to thank everybody who was important to the successful realization of thesis, as well as expressing my apology that I could not mention personally one by one. DEDICATION This thesis is dedicated to my late father, who taught me to stand firm and pursue my dreams. It is also dedicated to my late mother, who taught me to be humble and treat others with respect. LIST OF TABLES Table 1: Polyphenol content in vegetables (Source: Manach et al., (2004)…....12 Table2: Treatment arrangement 18for Nitrogen (N), Phosphorus (P) and potassium (K) trials……………………………………………………………………………….12 Table 3: Response of chemical concentrations in baby spinach to different levels of nitrogen (N) nutrition (dry weight basis……………..………………………39 Table 4: Response of chemical concentrations in baby spinach to different levels of phosphorus (P) nutrition (dry weight basis……………………………………..39 Table 5: Response of chemical concentrations in baby spinach to different levels of potassium (K) nutrition (dry weight basis………………………..………………39 Table 6: Response of chemical concentrations in baby spinach to different 8levels of nitrogen (N),

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Page | xvii LAN – Limestone Ammonium Nitrate Mg – Milligram Mg - Magnesium 3MS – Mean of Squares MMOL.M2-S – Millimol per meter square per second NM – Nanomoles N – Nitrogen NO3− – Nitrate Ns – Non Significant P – Phosphorus PCM – Protein Competition Mode Q - Quadratic RCBD – Randomized complete block design 3SAS – Statistical Analysis System Se - Selenium SOV – Source of Variance SS – Sum of Squares USA – Unites States of America WHO – World Health Organization Zn – Zinc ABSTRACT Baby spinach (Spinacia oleracea L.) is considered to be the one of the 1extremely nutritious vegetable, rich both in phytochemicals and core nutrients. Nowadays, phytochemicals30in plants are raising interest in consumers for their roles in the maintenance of human health. Variation in content of bioactive compounds and core nutrients is the main concern in vegetable production. Factors such as cultural practices specially fertilization, may affect the nutritional and medicinal Three parallel trials for NPK 3to investigate the response of baby spinach leaves to Nitrogen (N), phosphorus (P) and potassium (K) on chemical composition was conducted, with treatments arranged as follows: 0, 45, 75, 105, 120 kg·ha-1 N& P and 0, 63, 85, 106, 127, 148 9kg·ha-1 K in a randomized complete block design (RCBD) with four replications. The results demonstrated that,4application of nitrogenous, phosphorus, potassium fertilizers significantly increased the total phenols, total antioxidant activity , total flavonoid content & vitamin C, while magnesium, iron, zinc & selenium didn't respond. The increase in concentrations on total phenolic content, total antioxidant activity , total flavonoid content & vitamin C was observed, reaching maximum at 4345 kg·ha-1 N, 75 kg·ha-1 P& 85 kg·ha-1 K. The optimum rates of 4345 kg·ha-1 N, 75 kg·ha-1 P, 85 kg·ha−1 K were then used to formulate the NPK treatment combinations 9as follows: 0, 30: 30: 40, 45:45:60, 60:60:70, 75:75:90 kg·ha- 1, arranged in a randomised complete block design with three replicates. The results showed that total phenolic content, total antioxidant activity , total flavonoid content & vitamin C reached maximum in baby spinach leaves at N45:P45:K60 kg·ha-1. Keywords: baby spinach; bioactive compounds, minerals, core nutrition. CHAPTER 1 1. RESEARCH BACKGROUND ON BABY SPINACH 1.1 BACKGROUND Spinach is a leafy and 1extremely nutritious vegetable, rich both in core nutrients and phytochemicals. It 20is a vegetable that is provided fresh, frozen or canned to the consumer. Spinach harvested after a shorter growth period than normal is called baby spinach, and is marketed fresh to the consumer. This is a fairly new product that has turned out to be more and more popular in recent years because of its nutritional value (Hedges &Lister, 2007). 1Its nutrients comprise a range of vitamins and minerals, as well as 1phytochemicals. The major micronutrients in spinach are vitamins A (from β-carotene), C, K and folate, and the minerals, calcium, iron and potassium. The 1phytochemicals of most importance are the carotenoids, flavonoids, β-carotene, lutein and zeaxanthin and phenolic compounds (Bergquist, 2006). A number of studies have shown spinach to have strong antioxidant activity and high levels of antioxidant compounds such as phenolics and carotenoids (Hedges &Lister, 2007). Agronomic practices such as 8chemical fertilizers have made major contributions to improve crop yields and food nutrition (Fageria, 2009; Wang et al., 2008). There are many contributing factors in terms of fertilizer application that can influence the effectiveness of fertilizer such as the application method, application timing and the rate of application. All these factors must be investigated before fertilizer application to ensure effectiveness and to prevent problems associated with fertilizers. Problems such as under application and over application of fertilizers are seen to be challenging. On the other hand, excessive fertilizer application can have detrimental consequences. It 8can have adverse environmental effects on water quality, leaching, and runoff (Heckman, 2007; Heckman et al., 2003; Manotti et al., 1994; Sims, 1998; Sims et al., 1998). Consequently, it is imperative to investigate and determine fertilizer application rates that are beneficial and maximizes the 8yields while minimizing environmental contamination (Fontes et al., 1997; Heckman et al., 2003). The efficient use of fertilizers and optimal fertilizer management of Nitrogen, Phosphorus & Potassium are necessary to minimise production cost and to improve yield and quality (Fageria, 2009). Some studies are available 2on the identification of bioactive compounds in fruits and vegetables, pre-harvest and post-harvest factors. 12However, information on the effect of mineral fertilization on nutritional quality of baby spinach is scarce. 32As the consumption of fresh-cut spinach increases, it is important to investigate the good management practices, specifically fertilization and its effects on quality of baby spinach. The consumption of spinach increases because people are becoming health cautious and spinach is regarded as one of the healthy vegetables and rich in nutrients. The 64purpose of the present study was to investigate and quantify the variations on different bioactive compounds such as antioxidants, flavonoids, total phenolic content & carotenoids as well as the minerals (Magnesium, Zinc, Iron & Selenium) in baby spinach with reference to mineral nutrition. 1.2 RESEARCH PROBLEM Spinach 12is an important agricultural crop, not only because of its economic importance, but also for the nutritional values of its leaves, mainly due to the fact that they are an excellent source of nutrients and high level of phytochemicals. Spinach is increasingly becoming important in health because of its micronutrients and phytochemicals. Spinach has an additional advantage of being low in calories, which is very important in weight management. Therefore, it is becoming a food of choice to many people because of its nutritional importance. 30Nowadays, phytochemicals and antioxidants in plants are raising interest in consumers for their roles in the maintenance of human health. Phenolics and flavonoids are known for their health- promoting properties due to protective effects against cardiovascular disease, cancers and other disease (Kaur & Kapoor, 2001; Sardas, 2003) 12The intake of these compounds in foods is an important health protecting factor. They have been recognized as being beneficial for prevention of widespread human diseases, including cancer and cardiovascular diseases, when taken daily in adequate amounts (Kaur & Kapoor, 2001; Sardas, 2003). It is generally known that environmental factors and agricultural techniques have an effect on vegetables and fruits quality (Wang, 2006; Bafeel & Ibrahim, 2008). In particular, mineral fertilization influences antioxidant composition in some fruit and vegetables (Jeppsson, 2000; Kaur & Kapoor, 2001; Kopsell & Kopsell, 2006; Kemal et al., 2007). Some studies are available on the effect of32postharvest storage and processing on the antioxidant Page | 2 constituents (Flavonoids and Vitamin C) of Fresh-Cut Spinach (Marı´a I. Gil12et al., 2009). However, information on the effect of mineral fertilization on nutritional quality of Spinach is scarce. Variable content of bioactive compounds and minerals is the main problem in the production of vegetables, due to many factors especially fertilization. Consequently, the nutritional and medicinal value of vegetables may be affected. Producers often apply large quantities of fertilizer in an attempt to maximise yield and advance growth, which can result in the accumulation of nitrate in case of excessive nitrogen, reducing the quality of some vegetables. There are no results about the content of bioactive compounds and minerals in baby spinach grown on the principles of best management practices 3in South Africa. Currently the demand of baby spinach exceeds the supply and the applications of 22-45Nkg/ha, 22-45Pkg/ha and 63-138Kkg/ha have been reported in California, USA. These are the application rates from the growers since there is no scientific validation of these rates. 8Limited studies are available on the effect of nutrients on bioactive compounds of baby spinach and data that provide recommended rates such as N, P and K on quality have not been well established in South African conditions. Therefore, it was important to investigate the variation in concentrations of bioactive compounds (antioxidants, flavonoids, carotenoids and total polyphenols) and minerals (Magnesium, Iron, Zinc & Selenium) in baby spinach with reference to mineral nutrition. The main aim was investigate the effect of fertilization (N, P & K) baby spinach on their bioactive compounds. 12Additionally, any information on the physicochemical properties and bioactive compounds of baby spinach 12will provide a knowledge base that may be of some benefit to the production of baby spinach in South Africa. 1.3 MOTIVATION FOR MY RESEARCH 27Among the world's healthiest vegetables, spinach comes out as one of the vegetables that are rich in nutrients. 27Rich in vitamins and minerals, it is also concentrated in health-promoting phytonutrients such as carotenoids (beta-carotene, lutein, and zeaxanthin) and flavonoids to provide you with powerful antioxidant protection (Seddon et al., 1994). The benefits of spinach are many. Leafy green vegetables like spinach provide more nutrients than any other food, when compared calorie for calorie (Longnecker et al., 1997). 1Its nutrients include a range of vitamins and minerals (micronutrients), which can prevent deficiency diseases and are essential for normal physiological function, as well as phytochemicals1thought to help prevent chronic health problems such as cancer and heart disease, as well as other health problems associated with ageing (Hedges & Lister, 2007). In 25recent years, considerable attention has been directed towards the identification of natural antioxidants, namely those plant derived that may be used for human consumption regarding health promotion and disease prevention. It is vital to discover a method how we could grow healthy vegetables vegetable so that human body could have a higher intake of health-promoting bioactive compounds. This fact is very important in these days when we are exposed to lot of health related challenges. This study addresses such areas where the effect of mineral fertilization can be related to the concentration of health promoting phytochemicals. . One of the benefits of spinach is that it is readily available, just about all over the world and easy to prepare. The more interesting news about spinach, it is easy to find in the market or easy to grow and prepare. With its fine taste, baby spinach is a versatile food that can be easily included into a range of dishes. Unlike other vegetables it is harvested after a short period after planting. Spinach is a multipurpose food and important dietary vegetable. It can be eaten raw in a salad or it can be cooked (steamed or boiled) and eaten as a dish on its own or added to soups and other dishes. In the 32past few years, ready to use fresh-cut spinach has become more popular32(McGill et al., 1966; Burgheimer et al., 1967; Izumi2et al., 1997). Baby spinach was chosen for this study for two main reasons. It is becoming an increasingly popular product in South

Africa and elsewhere in the world. Spinach is known to be a healthy product and contains relatively high concentrations of bioactive compounds (Gil, Ferreres &Tomás-Barberan, 1999; USDA, 2005). In addition, baby spinach has the advantage of a short culture time and shelf life, making it an excellent model crop. Therefore, the 75objective of the study is to investigate the response of chemical composition and minerals in baby spinach to N, P, and K nutrition. 1.4 AIM OF THE STUDY 59The aim of this study is to investigate and determine the response of total phenolic content, total antioxidant activity , total carotenoid content, total flavonoid content & vitamin C as well as trace elements to 4Nitrogen (N), Phosphorus (P) and Potassium (K) nutrition. 1.5 OBJECTIVE OF THE STUDY 5.1.1 To determine rates/levels of application of N, P, and K fertilizers on the bioactive compounds in baby spinach. 5.1.2 To determine the interactions effect of NPK on the bioactive compounds in baby spinach. 1.6 HYPOTHESIS 6.1.1 Rate of applications of fertilizer N, P and K have no influence on the bioactive compounds in baby spinach. 6.1.2 No interactions effect on bioactive compounds in baby spinach after NPK application. 1.7 SCOPE AND LIMITATIONS OF THE RESEARCH Given the research aim and objectives of the study delineated above, the scope of the study entailed three aspects – rate of applications of fertilizer N, P & K, the 2variation in bioactive compounds in baby spinach as well the combined effect of N, P & K on the bioactive compounds. However, the study had a set of limitations inherent in the topic investigated. Firstly, not all varieties of baby spinach could be investigated for this research, given the nature of the research methods used and the time-frames selected. Secondly, the study was conducted on the protected environment and the effect of this was not taken into consideration in this study. Lastly, only one method of fertilizer application was used in this study which was side dressing method. 1.8 ORGANISATION OF THE THESIS The thesis is presented in four chapters. Chapter 1 provides the research background on baby spinach, the importance and research problem of the study. An outline of brief research motivation, aim and objectives, scope and limitations of the study is also discussed. Chapter 2 consists of a review of the relevant literature pertaining to baby spinach. Chapter 3 provides a brief description of study areas, research design and methods, as well as data collection and analyses. The thesis ends with Chapter 4 which deals with set of research findings, with particular reference to the mineral nutrition and bioactive compounds in baby spinach. The chapter details the analyses and discussion of the variation in 2concentrations of bioactive compounds in baby spinach. The chapter also provides a summary of research findings, concluding remarks and recommendations which set research goals for further research. CHAPTER 2 2. LITERATURE REVIEW ON MINERAL NUTRITION AND QUALITY OF BABY SPINACH 2.1 INTRODUCTION This chapter outline and review literature. The review of literature includes the botany of baby spinach, composition of baby spinach, health benefits as well as mineral nutritional of baby spinach. To end the chapter, a brief summary of these sections is also provided. 2.2 THE HISTORY AND BOTANY BABY SPINACH 2.2.1 Origin and distribution Spinach is a leafy plant which was first planted for medicinal purposes but was eaten by monks on feast days by 1551 AD (SADAFF, 2010). It originates in Iran and soon found its way into Africa. From there it spread to Europe and now it is widely grown all over the world. (Asai et al., 2004). It was probably introduced into Europe during the Middle Ages; brought to North America by European settlers (Asai et al., 2004). 2.2.2 Botanical Description of baby 54spinach Spinach (Spinacia oleracea) is an edible flowering plant in the family of Amaranthaceae (FAO, 2008). 15Common spinach, Spinacia oleracea, was long considered to be in the Chenopodiaceae family, but in 2003, the Chenopodiaceae family was combined with the Amaranthaceae family under the family name 'Amaranthaceae' in the order Caryophyllales. Within the Amaranthaceae family, Amaranthoideae and Chenopodioideae are now subfamilies, for the amaranths and the chenopods, respectively.15It is an annual plant, which grows to a height of up to 30 cm (Boswell, 1949). Spinach is dioecious; plants generally produce 36either male (staminate) or female (pistillate) flowers but only one sex is to be found on any one plant so both male and female plants must be grown if seed is required and are pollinated by wind. The plant not is self-fertile (Asai et al., 2004). 2Baby spinach is harvested after a shorter growth period and sown at closer density than regular spinach and thus the leaves are smaller, hence the name.2Spinach is a long-day plant that is prone to bolting during the summer (Bergquist, 2006). The bolting tendency varies between cultivars, and cultivars that are less prone to bolting could be used during the earlier part of the summer. For baby spinach, bolting is often not a problem because the leaves are harvested early (Respondek & Zvalo, 2008). 2.2.3 15Types of spinach A distinction can be made between older varieties of spinach and more modern ones. Older varieties tend to bolt too early in warm conditions. Newer varieties tend to grow more rapidly, but have less of an inclination to run up to seed (Rolland & Sherman, 2006). The older varieties have narrower leaves and tend to have a stronger and bitterer taste. Most new varieties have broader leaves and round seeds (Rolland & Sherman, 2006). Spinach cultivars are generally categorized according to the leaf blade variations and are classified as savoy have wrinkled leaves, semi-savoy have slightly crinkled leaves , and flat or smooth leaved lacks wrinkles and are flat (Grieve, 1971; Respondek & Zvalo, 2008). 2.3 COMPOSITION AND QUALITY OF BABY SPINACH 2.3.1 MICRO NUTRIENTS Spinach has 28high concentration of vitamin A, E, C, and K, and also folic acid, oxalic acid. Along with these chemicals various minerals present in the spinach such as magnesium, manganese, calcium, phosphorus, iron, zinc, copper and potash. (Mehta and Belemkar, 2014). 69Spinach is an excellent source of chlorophyll, beta carotene (needed for the production of vitamin A), riboflavin, sodium and potassium. It is great food for the vegetarian (Baby-Spinach-nutrition-information.com). 1Other nutrients present in smaller quantities include some B vitamins – thiamine (B1), riboflavin (B2) and B6 (Hedges & Lister, 2007). 2.3.1.1 Magnesium Magnesium (Mg) is one of the minerals present in the spinach (Deven Mehta et al 2014). It 55is part of the chlorophyll molecule existing in all green plants and is, consequently, important for photosynthesis and also helps to stimulate numerous Page | 8 plant enzymes required for development (Plaster, 2003; Gardiner & Miller, 2004). Magnesium deficiency symptoms appear first in older leaves as chlorosis (Acquaah, 2002). It was reported by Mengel & Kirby, (1982) that shortage of magnesium impedes protein synthesis In humans, mmagnesium is an essential mineral needed for survival. The health advantages of magnesium are extensive. A number of studies have previously shown magnesium to have more health benefits. Among many functions, magnesium can benefit people in blood pressure; help prevent heart attack and stroke. It is also vital for good bone and cartilage formation among others (Zeng, 2010). 2.3.1.2 70Iron Spinach is known to be the source of Iron (Hedges & Lister, 2007). Iron is important for the synthesis of chlorophyll, which gives rise to the green colour of plants (Aquaah, 2002). Iron shortage shows up as interveinal chlorosis of young leaves and chlorosis spreads to the older leaves as the severity of the deficiency increases (Jones, 2003. According to Zeng, (2010), iron plays a key 53role in strengthening the immune system of the human body by making it strong enough to fight off infections. It also is important for the proper growth and development of the human body. 2.3.1.3 Zinc Zinc is an essential trace element present in spinach (Deven Mehta et al 2014). In plants, zinc helps with growth hormones, seed production and maturation, and starch formation (Brady & Weil, 2004). If there are shortages of zinc, plant leaves are severely reduced in size, while the internodes shorten to give a rosette appearance and the leaves become mottled (Acquaah, 2002:190; Gardiner & Miller, 2004). In humans, zinc is found in all parts of the body: it is in organs, tissues, bones, fluids and cells. Muscles and bones contain most of the body's zinc (90%). The known health benefits of Zinc include proper functioning of the immune and digestive systems, control of diabetes, reduction of stress levels, energy metabolism, and an increased rate of healing for acne and wounds (Zeng, 2010). 2.3.1.4 Selenium Selenium is a trace element that is essential human health in small amounts. Spinach is one of the green leafy vegetables which contain selenium as one of the minerals. Selenium plays a key role in the metabolism. Selenium has is significant in health 47because of its antioxidant properties. Antioxidants protect cells from damage. There is some evidence that selenium supplements may reduce the odds of prostate cancer (Hatfield et al., 2012). 2.3.2 PHYTOCHEMICALS Phytochemicals are compounds that are produced by plants. 76They are found in fruits, vegetables, grains, beans, and other plants. With most greens like spinach, when eaten raw it is loaded with a high vitamin and mineral content, but when cooked it releases many phytochemicals. Phytochemicals are natural plant organic compounds often called phytonutrients that are well known for potential health benefits. The 1phytochemicals of most importance in baby spinach are the carotenoids, β-carotene, lutein and zeaxanthin, along with phenolic compounds. Other phytochemicals include chlorophyll, glutathione, α-lipoic acid and betaine (Joseph et al., 2002). 2.3.2.1 Total antioxidant activity content Antioxidants are 34chemicals often found naturally in plant foods, which can help protect our body cells from being damaged by 'free radicals'. Free radicals are produced both naturally in our body and due to exposure to pollutants and result in cell damage. Antioxidants7are naturally present in fruits and vegetables. They possess 7ability to neutralize free radicals or their actions (Cadenzas & Packer, 1996; Nicoli et al., 1999). The 7positive health effects of fruit and vegetable have been credited to the fairly high antioxidant concentration in fruits and vegetables (Ames et al., 1993; Rice - Evans & Miller, 1995). Antioxidants are naturally present in fruits and vegetables. They possess 7ability to neutralize free radicals or their actions (Cadenzas & Packer, 1996; Nicoli et al., 1999). Free radicals have been implicated in the aetiology of several major humans' ailments, including cancer, cardiovascular disease, neural disorders, diabetes and arthritis (Sies, 1996; Yoshikawa et al., 2000; Devasagayam et al., Page | 10 2004). Antioxidants are found in most vegetables, fruits and green tea. As a rule, dark-coloured fruits and vegetables have more antioxidants than other fruits and vegetables. 1Several studies have examined the antioxidant activity of spinach and other vegetables. Cao et al., (1996) gave spinach an antioxidant score of 17(based on ORAC scores using three different radicals), rating it third of the 22 vegetables examined.1Similarly, Pellegrini et al., (2003) ranked spinach first; eighth and first respectively out of 31popular vegetables as measured by the Ferric- Reducing Ability of Plasma (TRAP), 1Total Radical-trapping Antioxidant Parameter (TRAP), and Trololx Equivalent Antioxidant Capacity1(TEAC) antioxidant assays. A similar result was found by Wu et al., (2004) & Chu et al., (2002). The latter not only ranked spinach extremely highly for antioxidant activity but also in terms of antiproliferative activity. By contrast, however, spinach was placed towards the middle of the field of 20 common vegetables in another recent study (Chun et al., 2005). 2.3.2.2 Total phenolic content 6Phenolic compounds are commonly found in both edible and non-edible plants. They have been reported to have multiple biological effects, including antioxidant activity crude extracts of fruits, herbs, vegetables, cereals, and other plant materials rich in phenolics in 6Table 1 below (Manach et al., 2004). Phenolic are progressively of interest in the food industry because they retard oxidative degradation of lipids and thereby increase the quality and nutritional value of food (Khkönen et al., 1999).1Chemical structures range from quite simple compounds such as caffeic acid to highly polymerised substances such as tannins. There are many diverse groups of phenolics but the most common phenolics found in foods are generally phenolic acids, flavonoids, lignans, stilbenes, coumarins and tannins (Harbourne, 1993). Phenolic25compounds have received considerable attention for being potentially protective factors against cancer and heart diseases, in part because of their potent antioxidative properties and their ubiquity in a wide range of commonly consumed foods of plant origin (Deven Mehta and Belemkar, 2014). 6Table 1: Polyphenol content in vegetables (Source: Manach et al., (2004). Chemical compositions Source (or mg L−1) 6By wt or vol mg kg− 1fresh wt6Flavonols Yellow onion 350–1200 Quercetin Curly kale 300–600 Kaempferol Leek 30–225 Myricetin Cherry tomato Broccoli Beans, green or white Tomato 15–200 40–100 10–50 2–15 Flavones Apigenin Luteolin Daidzein Catechin Sinapic acid Parsley Celery Capsicum pepper Soybeans, boiled Beans Potato 240–1850 20–140 5–10 200–900 350– 550 100–190 2.3.2.3 Total flavonoid content content 2Flavonoids are compounds with yellow to white or blue, purple to red colour2(Greenberg et al., 1996; Kolb et al., 2001).2Bioavailability of flavonoids differs extensively, and most likely depends on influences such as food matrix; human intestinal micro flora and flavonoids structure acid (Manach et al., 2005). The bioavailability of flavonoids is most probably lower than that of some other antioxidants, such as ascorbic (Berquist, 2007). Epidemiological study 20on flavonoids specified that a high dietary intake is related with a lower risk of coronary heart disease3(Hertog et al., 1995 & Knekt et al., 1996) dementia (Commenges et al., 2000) and some form of cancer (Neuhouser et al., 2004). Flavonoids are found in lots of grains, vegetables, and fruits. Spinach is 28very rich in the flavonoids. Various flavonoids are reported to be present in spinach (Mehta et Page | 12 al., 2014). The concentration of 20flavonoids in vegetables and other plants differs depending on influences 3such as genotype, environmental growing conditions, growth stage, postharvest handling, and storage conditions3(Patil et al., 1995 and Howard et al., 2002).20These factors affect both total flavonoid content concentration and the composition of flavonoids may consequently result in variation in flavonoids content. According to Fico et al., (2000) & Vogt et al., (1994), the concentration in flavonoids and 20composition may also change during plant growth.6Flavonols Flavonols are the most abundant flavonoids in foods, and the main representatives are kaempferol and quercetin (Manach et al., 2005). Considered as the most abundant dietary flavonol, quercetin is a potent antioxidant because it has all the right structural features for free radical scavenging activity. They are generally present at relatively low concentrations of ∼15–30 mg kg−1 fresh weight. According to Manach et al., 2002, 6the richest sources are onions (up to 1.2 g kg−1 fresh weight), curly kale, leeks, broccoli, and blueberries. Flavones In 6fruit and vegetables flavones are much less common than flavonols. Parsley and celery are identified as important sources by 6Manach et al., 2005. Cereals such as millet and wheat contain C-glycosides of flavones (Boyle et al., 2000& Erlundet al., 2000). Large quantities 6have been identified on the skin of citrus fruit (Nielsen et al., 2003).6Flavanones In human foods, flavanones are found in tomatoes and certain aromatic plants such as mint, but they are present in high concentrations only in citrus fruit. The main aglycones (the non-sugar component that results from hydrolysis of glycoside flavanones) are naringenin in grapefruit, hesperetin in oranges, and eriodictyol in lemons (Hertog et al., 1993). 60Isoflavones Isoflavones are provided only by soybean-derived products. They49are found almost exclusively in leguminous plants. Soya and its processed products are the major sources of isoflavones in the human diet6(Hollman et al., 1996; Moon et al., 2000). Flavanols Flavanols are present in both the monomer form (catechins) and the polymer form (proanthocyanidins). Catechins are found in many types of fruit such as apricots, which6are the richest source (Manach et al., 2005). A drink of 6green tea comprises up to 200 mg catechins (Day et al., 2001).6Catechin and epicatechin are the main flavanols in fruit, whereas gallocatechin, epigallocatechin, and epigallocatechingallate are found in certain seeds of leguminous plants, in grapes, and more importantly in tea (Manach et al., 1998; Wittig et al., 2001). 2.3.2.4 Total 28carotenoids Spinach shows presence of different carotenoids like lutein, -carotene, violaxanthin and 9'-(Z)-neoxanhin (Mehta et al., 2014). Lutein and zeaxanthin 1Spinach contains some of the largest amounts of lutein and zeaxanthin from vegetable sources. Although kale contains more, it is not commonly eaten and is therefore not a major food source. Studies have shown that these compounds are selectively accumulated in different parts of the eye, where they are by far the most abundant of the major carotenoids present (Hedges & Lister 2007). This has led to the suggestion that they may be important in protecting against age-related eye problems, particularly macular degeneration and the formation of cataracts. There is some epidemiological evidence to support this (Sies & Stahl 2003; Mares-Perlman et al., 2002).1The fact of their antioxidant activity has led to assumption that these carotenoids, particularly lutein, which is more widely dispersed in the body, could protect against diseases such as cancer and cardiovascular disease as well as boosting the immune function (Hedges & Lister, 2007). Epidemiological research on the influence of these particular carotenoids on site-specific cancers is relatively new and sparse. The most promising areas of research would appear to be in relation to skin cancer (in combination with other carotenoids) (Slattery et al., 2000)1and breast cancer (Mares-Perlman et al. 2002). In relation to 1cardiovascular disease (CVD), studies have found high serum levels of lutein and zeaxanthin to be associated with a reduced risk of coronary heart disease (Dwyer and Navab, 2001).1Additionally, the consumption of green leafy vegetables (which also contain lutein and zeaxanthin) was associated with a reduced incidence of stroke (Joshipura et al., 1999). 2.3.2.5 Vitamin C 27Research has shown that spinach leaves that look fully alive and vital has greater concentrations of vitamin C than spinach leaves that are pale in color (Edenharder et al., 2001). 45The benefits of vitamin C may include protection against immune system deficiencies, cardiovascular disease, prenatal health problems, eye disease, and even skin wrinkling. Vitamin C48is rapidly finding new applications in protecting against endothelial dysfunction, high blood pressure, and the blood vessel changes that precede heart disease. (Rossig et al., 2007; Fotherby et al., 2000). 2.4. OTHER PHYTOCHEMICAL IN BABY SPINACH 2.4.1 Glutathione Glutathione is an important antioxidant in plants, animals, fungi and some bacteria and archaea, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides (Pompella et al. 2003) 1An extremely important endogenous antioxidant (synthesised within the body), glutathione is relatively rare in foods. One of its major functions is to protect DNA from oxidation, but it also detoxifies carcinogens, boosts the immune system, supports liver health and reduces inflammation (Joseph et al. 2002). 2.4.2 α-Lipoic acid Like glutathione, α-lipoic acid is a vital antioxidant largely synthesised in the body, but also present in some foods. It is important for energy metabolism and its antioxidant activity may protect against chronic diseases. In addition, it may assist memory (Joseph et al. 2002). According to Berkson (1998), it is an exceptional antioxidant that may slow aging, repair liver damage and diminish the risk of heart disease, diabetes and cancer. 2.4.3 D-Glucaric acid D-Glucaric acid (GA) is a nontoxic, natural compound (Walaszek et al., 1997). 1It is believed that D-glucaric acid may lower blood cholesterol (Joseph et al., 2002) 2.3.4 Coenzyme Q10 Another endogenous compound, coenzyme Q10 is a critical component in energy metabolism, but also acts as an antioxidant in cell membranes and lipoproteins. The best food sources are meat, fish and oils, but spinach is one of the best vegetable sources.1It is thought that it may prevent cardiovascular disease by lowering levels of homocysteine, a compound associated with the development of heart disease (Joseph et al., 2002). 2. 3.5 Chlorophyll content

1Relatively little is known of the health effects of chlorophyll, the pigment that causes the green colour in plants and is the primary photosynthetic compound. Some research suggests that it may be important in protecting against some form of cancer, as it is thought that the chlorophyll binds to mutant DNA and prevents it proliferating. As it is high in 1concentrations in so many edible plants, it may some of the protective effects observed with diets rich in green vegetables (Fahey et al., 2005). 2.5 THE HEALTH BENEFITS OF BABY SPINACH 2.5.1 Introduction 2Fruit and vegetables contain a wide range of substances that are suggested to be part of these health-enhancing effects. Numerous 66studies have been conducted in regard to the health benefits of fruits and vegetables. 2There is strong evidence that a diet rich in fruit and vegetables has a positive result on human health, offering protection against degenerative diseases of ageing, such as heart disease, cardiovascular disease, Alzheimer's disease, cataracts and several forms of cancer (Williamson, 1996; Liu et al., 2000; Gandiniet al., 2000; Liu et al., 2001; Joshipura et al., 2001; Kang et al., 2005). Fruits and vegetables are known for being rich in both micronutrients and phytochemicals (Hedges &Lister, 2007). Important 40micronutrients such as vitamins, minerals and trace elements, fruit and vegetables contain other compounds that may have a positive result on human health. And also phytochemicals such as carotenoids, flavonoids and other polyphenols, phenolic acids, glucosinolates, allylic sulphides, isothiocyanates, dietary fibres, phytosterols and monoterpenes (Lister, 1999; Kris-Ethertonet al., 2002). These are the health benefits shown by different human and animal studies in relation to spinach consumption: 2.5.2.1 Protects against aging and Improves brain function - Spinach protects brain function from premature aging and slow old age's typical negative effects on mental capabilities by preventing the harmful effects of oxidation on brain (Wang et al., 2005). - One of the major health benefits of spinach is credited to carotenoids compounds, those are 1lutein and zeaxanthin, is that of protecting against eye diseases such as macular degeneration (gradual loss of central vision, associated with old age). More studies also indicated that spinach, spinach extracts, and spinach compounds may delay or retard age-related loss of brain function, reduce the extent of post-ischaemic stroke damage to the brain. (Hedges & Lister 2007). 2.5.2.3 Cancer-fighting antioxidants - As the spinach is rich in vitamin A and flavonoids and those compounds are 10known to help the body protect from lung and oral cavity cancers. Moreover, spinach leaves are believed to protect human body from cancers of colon and prostate (Byers & Perry, 1992). A study was conducted of New England women demonstrated 3less breast cancer cases among those who ate spinach on a regular basis. And again a study in laboratory animals shown that spinach extracts can reduce skin cancer and potential in slowing stomach cancer as well. (Gates et al., 2007; Longnecker et al, 1997; Williamson, 1996). 2.5.2.4 Promotes healthy heart - Spinach is an excellent promoter of cardiovascular health (Blomhoff, 2005). 10Regular consumption of spinach in the diet helps protect human body from cardiovascular diseases (The antioxidant properties of spinach work collectively, to support good cardiovascular health by preventing the harmful oxidation of cholesterol (Blomhoff, 2005; Lucarini et al., 2006). 2.5.2.5 Assist in fetal development - Spinach 10contains good amounts of many B-complex vitamins such as vitamin- B6 (pyridoxine), thiamin (vitamin B-1), riboflavin, folates and niacin. Folates help prevent neural tube defects in the offspring (Bernhoft, 2010; Hine, 2003; van de Veyver, 2002). - 10It is also good source of omega-3 fatty acids. Enough 35consumption of omega-3 fatty acids is vitally important for pregnant woman as they are critical building blocks of fatal brain and retina. Omega-3 fatty acids may also play a role in determining the length of gestation and in preventing perinatal depression (Jensen, 2006). 2.5.2.6 Promotes healthy bodies - Spinach leaves are an excellent source of 10vitamin K. Vitamin K plays a vital role in strengthening the bone mass by promoting osteotrophic (bone building) activity in the bone (Pearson, 2002; Shearer, 2009). 10Regular consumption of spinach in the diet helps prevent osteoporosis (weakness of bones), iron- deficiency anemia (Bernhoft, 2010). - Spinach has vitamin C is, 10which helps the body develop resistance against infectious agents and scavenge harmful oxygen-free radicals. It also contains a good amount of minerals like potassium, manganese, magnesium, copper, iron and zinc. Potassium is an important component of cell and body fluids that helps controlling heart rate and blood pressure. Manganese and copper are used by the body as a co-factor for the antioxidant enzyme, superoxide dismutase (Pearson, 2002). Copper is required in the production of red blood cells. Zinc is a co-factor for many enzymes that regulate growth and development, sperm generation, digestion and nucleic acid synthesis. Magnesium in spinach works toward healthy blood pressure levels 10Iron is an important trace element required by the human body for red blood cell production and as a co-factor for oxidation-reduction enzyme, cytochrome- oxidase during the cellular metabolism. (Jensen, 2006 & Zeng, 2010). 2.7 THE GROWING OF SPINACH 2.6.1 Seeding/Planting 5Spinach is commonly sown into rows spaced 20 - 30 cm apart. In recent years, growers have experimented with spinach being grown in rows 5 cm apart and seed spaced 5 cm apart in the row. Spinach is seeded on raised beds with 10-20 rows in the bed. A bed that is raised a few centimetres will aid in air and water drainage. For the baby spinach market, commercial growers have been experimenting with seeding in rows spaced 5 cm apart and seeds spaced 1 – 1.5 cm apart in the rows. 2-5 rows of spinach are grown on raised beds which could be 50-100 cm wide (Respondek & Zvalo, 2008). 2.6.2 Soils Spinach grows well on a variety of soils, although fertile, sandy loams with high organic matter content are preferred. Spinach grows well 5on well drained sandy loams or loams high in organic matter. Soil sampling and testing must be done before planting and regularly to determine the soil ph. 5Spinach is not tolerant to acidic soils; therefore5soil pH should be between 6 and 6.8. Spinach is not tolerant to acidic soils, therefore it is recommended to take samples regularly to ensure the right soil ph. 5Soils with good drainage and that warm up early in the season are recommended for spinach especially 5early and over wintered crops should be planted on such soils (SADAFF, 2010). 2.6.3 Irrigation 9Baby spinach has a relatively shallow root system and thrives on frequent, short irrigations to maintain uniformly moist soil for maximum leaf production (Berqguist, 2006).5Spinach requires a regular supply of moisture since it is a shallow rooted crop and should receive 25 mm of water every five days from rainfall or irrigation. The first five days of plant growth5(germination and seedling emergence) are very moisture dependant and require 5 mm-12 mm of water. Sprinkler irrigation is used on spinach production (Respondek & Zvalo, 2008 Joseph et al. 2002). 2.6.4 Fertilization 33Fertilization is one of the most practical and effective pre-harvest methods to regulate and increase yield and nutritional quality of crops for human consumption (Fallovo, 2009). 5Recommendations for supplemental organic matter, fertilizer, lime or manure should be based on a soil test and a nutrient management plan.5Nutrient management plans balance the crop requirements and nutrient availability, with the aim to optimize crop yield and minimize ground water contamination, while improving soil productivity. If spinach is stressed by a lack of nutrients, vegetative growth is retarded and the plants are more prone to bolting (Respondek & Zvalo, 2008). Fourteen 17mineral nutrients are classified as either macronutrients or micronutrients based on their plant requirements. There are six macronutrients: Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S). The macronutrients, N, P, and K, are often classified as 'primary' macronutrients, because deficiencies of N, P and K are more common than the 'secondary' macronutrients, Ca, Mg, and S. The micronutrients include boron (B), chlorine (Cl), copper (Cu), iron (Fe), manganese (MN), molybdenum (Mo), nickel (Ni) and zinc (Zn). Most of the macronutrients represent 0.1 to 5%, or 100 to 5000 parts per million (ppm), of dry plant tissue, whereas the micronutrients generally comprise less than 0.025%, or 250 ppm of dry plant tissue (Wiedenhoeft, 2006). 2.6.4.1 Nitrogen

18Nitrogen is used by the plant to produce leafy growth and formation of stems and branches. Plants most in need of nitrogen include grasses and leafy vegetables such as cabbage and spinach. Basically, the more leaf a plant produces, the higher its nitrogen requirement. (Zeng, 2010). Nitrogen supply is one of the major environmental factors that control plant mechanisms and is closely correlated to crop quality (Okazaki 68et al., 2008). The effects of N in spinach on the content of nitrate, oxalic acid, vitamin C and other antioxidants 52has been reported in previous studies (Elia et al., 1998; Zornoza & Gonzalez 1998; Logan et al., 1999; Santamaria et al., 1999; Ter Steege et al., 1999). The management of nitrogen fertilizer supply is important, particularly to ensure the adequate levels for normal plant growth and the levels that are suitable for human consumption (Maereka, 2007) as per WHO standards. Baby 11spinach is a short – season crop that is harvested when the crop is young. As a result, the nutrient uptake is relatively low. For instance, the nitrogen (N) content of spinach may vary from 20 to pounds of nitrogen per acre (22 to 45 kg·ha- 1). Spinach is a medium – heavy nitrogen feeder (Hedges & Lister, 2007). Nitrogen encourages leaf growth so it is useful for lawns, houseplants, spinach or other leafy vegetables. Nitrogen supplied during growth greatly increases the size and qualify of your spinach. Good leave coverage is also important for photosynthesis so virtually all plants need nitrogen but too much can make a plant soft (Cao et al., 1996). Autumn 11application of nitrogen is not recommended due to the risk of NO_3 -N leaching beyond the root zone by the winter rains. Small quantities of nitrogen, 20 pounds per acre (22 kg·ha- 1) are applied before planting or at planting, an additional top dress or water – run application of 20 to 30 pounds of nitrogen per acre (22 to 34 45kg ·ha- 1) is generally sufficient for baby spinach production.11Soil nitrate levels greater than 20 parts per million in the top 15 cm are adequate for crop growth (Williamson, 1996). 19Increased additions of N usually result in increased yield of crop plants (Greenwood et al., 1980; Szwonek, 1986; Mills & Jones).19However, toxicity from excessively high N concentrations is possible. Severe yield decrease was reported when cabbage (Brassica oleracea L. var. Capitata) was grown at elevated N rates (602 mg N L-1; Huett, 1989). 2.6.4.2 Phosphorus 18Phosphorus is essential for seed germination and root development. It is needed particularly by young plants forming their root systems and by fruit and seed crops. Root vegetables such as carrots, swedes and turnips obviously need plentiful phosphorus to develop well (Zeng, 2010). 3Levels above 60 parts per million (ppm) are adequate for spinach growth, for soils below this level, especially in the winter, pre-plant applications of 20 to 40 pounds per acre of P_2O_5 (22 to 45 kg ·ha- 1) or 3applications of 20 pounds per acre of P_2O_5 at (22 to 45kg·ha- 1) planting are recommended (Joseph et al., 2002). Phosphates are needed for healthy root growth in seedlings in baby spinach (Colomb et al. 2000). 2.6.4.3 Potassium Potassium is 18used in the process of building starches and sugars so is needed in vegetables and fruits (Zeng, 2010). 9Potassium is one of the principle plant nutrients important for yield production and quality determination of crop (William, 2007). Potassium in the form of form of potash encourages flowering, fruiting and good colour (Bergquist, 2006). 2.6.4.3 Lime 5On acidic soils, spinach will have low germination, yellowing and browning of the leaf tips, the roots will burn and growth of the plant will be slowed. If the pH is too high, chlorosis may result on leaves5Lime should be applied to maintain the soil pH in the range 6.5 to 6.8. Spinach is very sensitive to soil acidity, therefore it is recommended to get soil tested on a regular basis to ensure the maintenance of pH in the acceptable levels (Respondek & Zvalo, 2008). 2.6.5 Harvest Harvesting for baby spinach can be started as early as a month after sowing, few leaves from each plant can be harvested. Bigger leaves can be pinched off right at the base of the plant, leaving the smallest leaves for next harvest. Leaves can be harvested when they are 3-4 inches tall at 35 days after planting (USDA, 2005; Gandiniet al., 2000). The entire plant can be harvested when it reaches maturity, just prior to bolting. The time of harvest is crucial as there may be variations in the concentrations on chemical composition during the day as result of pre-harvest factors such as water availability and light intensity (Mozafar, 1994). Harvested spinach can be kept in a moisture-retentive container in the refrigerator for as long 40-50 days (Gandiniet al., 2000). 2.6.5.1 11Postharvest handling Spinach is fairly perishable and will yellow when stored at higher than recommended temperatures. Spinach must be store in low temperatures of 0°- 5° Lucier et al., (2004). 3Lower temperature has positive effect on ascorbic acid and carotenoids content (Kalt, 2005). The content of ascorbic acid in spinach rapidly 3decreases during storage at ambient temperature (Beuscher et al., 1999).11However, the main cause of postharvest losses is decay related with mechanical damage during harvest and postharvest processes. Spinach has a large surface-to-weight ratio and a very high respiration rate, it should be cooled rapidly to prevent excessive weight loss and wilting. Spinach11is sensitive to ethylene (increases yellow and may increase decay) and moderately sensitive to freezing injury after harvest (Pellegrini et al., 2003; Respondek & Zvalo, 2008). 5Spinach can be held 10 to 14 days at a temperature of 0°C and a relative humidity of 95% to 100%. Wilting, yellowing of leaves and decay are likely to occur after 10-14 days in storage (Pellegrini et al., 2003). 5Spinach is very sensitive to ethylene and should not be stored or transported with apples, melons or tomatoes because accelerated yellowing with result (Respondek & Zvalo, 2008). 2.7. THE VARIATIONS IN CONCENTRATION OF BIOACTIVE COMPOUNDS 2.7.1 Introduction The variations in 81concentration of bioactive compounds in fruit and vegetables have been observed in numerous studies. The concentration of 2bioactive compounds in fruits and vegetables are definitely changing and are influenced by numerous pre- and postharvest factors (Berquist, 2006). Factors such as cultivar, soil type, fertilization, irrigation, the 1degree of maturity at harvest, harvesting, storage, method of processing (Hedges & Ledger, 2007; Kirakosyan et al., 2004). Variations can be 2higher or lower concentrations of a certain compound as a result of these factors. (Berquist, 2006). The following are the factors affecting or have the influence 2on the concentrations of bioactive compounds in spinach. 2.7.2 Factors affecting concentrations of bioactive compounds in baby spinach leaves 2.7.2.1 Cultivar The 1fact of inter-cultivar variation in general is well established.1A study of 11 commercial lines and 15 breeding lines showed large variations in antioxidant activity and phenolic content (Hedges & Lister, 2007). Variations were also notable by growing season, with significantly higher levels of antioxidant activity and phenolic content in over-winter spinach. (Howard et al., 2002). 2.7.2.2 Climatic requirements 2Climatic conditions have a strong effect on the concentration of bioactive compounds (Weston & Barth, 1997). Climatic factors differ with growing site, during the season (Howard et al. 2002). 2Temperature, both in terms of total or average temperature and the extremes during the growth period, may influence the chemical composition (Weston & Barth 1997; Lefsrud et al., 2005). 2.7.2.3 Genetic variation Another 1study also showed phenolic content variation according to genotype, as well as level of maturity, with quantities significantly higher at the mid-maturity stage (Pandjaitan et al., 2005). Genetic 2factors thus have a large influence on the content of bioactive compounds in fruit and vegetables. Variation between cultivars of the same species may also be large (Mercadante & Rodriguez-Amaya, 1991; DuPont et al., 2000; Howard et al., 2002). Ascorbic acid is found in 2vegetables in widely varying concentrations (Lee & Kader, 2000). 6.7.2.4 Agronomic practices Agronomic practices such as nutrient and water availability also have 2influence on the bioactive compounds (Weston & Barth, 1997). There are many contributing factors in terms of fertilizer application that can influence the 2concentrations of bioactive compounds. To predict the response of bioactive compounds to nitrogen fertilizer may be complicated. The results may show 2both increasing and decreasing concentrations due to fertilisation. This may be depending on how plant growth responds to the fertilisation2(Mozafar, 1993). Nitrogen fertilisation may also enhance foliage, reducing light intensity reaching shaded parts of the plant, which may affect the concentration of the bioactive compounds (Mozafar, 1994; Lee & Kader, 2000). 2.7.2.5 Method of processing 1Processing such as freezing also influence the nutrients in spinach. The amounts of some nutrients such as vitamin C and folate are reduced with freezing, but others, such as β-carotene, lutein and zeaxanthin, are improved (Hedges & Ledger, 2007). Cooking has also influence over nutrients in spinach. Cooking has both positive and negative effects on nutrients (Joseph et al. 2002. It can reduce vitamin C and folate, on the other hand it can increase the bioavailability of total 1carotenoids like β- carotene and lutein and zeaxanthin. Light cooking or steaming is often recommended to enhance carotenoid bioavailability while minimising the loss of other nutrients (Joseph et al. 2002). 2. 7.2.6 2Harvesting factors There is considerable variation in concentration of bioactive compounds during growth and maturation of fruit and vegetables. The variation is most likely especially obvious in fruit ripening, where the carotenoid or flavonoid provide the colour of the ripe fruit (Kalt, 2005).2Harvesting baby spinach leaves early, while they are slightly smaller than at conventional harvest may give increased concentrations of bioactive compounds.2Harvesting later than the current conventional time may give a higher yield, but the concentrations of bioactive compounds may not be improved although some compound are 2likely to be higher at a later harvest (Bergquist, 6002). 2Timing of harvest during the day is also of significance, as there may be great variation in the concentrations during the day, probably related to water content (when concentrations are given on a fresh weight basis) or to light intensity (Mozafar, 1994; Veit et al., 1996). 2.7.2.7 Storage Storage environment 2after harvest may also have a big impact on the bioactive compounds (Kalt, 2005). 2Storage not only affects the concentration of these compounds, but the concentration of these compounds may affect storability as well.2Baby spinach is generally sold fresh in polypropylene bags and the maximum storage time is about 10 days (Lucier et al. 2004; Bergquist, 2006). 2Storability can be significantly enhanced by lowering temperatures, increasing humidity and by modifying the surrounding atmosphere (Wills et al., 1998). Temperature must be managed well during storage as it can results in deterioration of baby spinach. 2Management is crucial for keeping the quality of harvested fruits and vegetables, both in terms of appearance and biochemical composition.

A lower temperature decreases metabolic rates and thereby slows down deterioration. But a temperature that is too low may also impose problems. 2Recommended storage temperature is close to 0 °C2(Wills et al., 1998). The storage period is also of major importance, since the concentrations change over time. Light conditions during storage generally affect concentrations of these compounds as well, although in different ways. Photosynthesis may be supported in light-stored leaves, and light storage may also increase ascorbic acid synthesis (Toledo et al., 2003). Carotenoids, on the other hand, may decrease to a higher extent in vegetables stored in light (Kopas-Lane & Warthesen, 1995).

2.8 BRIEF SUMMARY OF THE LITERATURE REVIEW Spinach is known for being nutritious vegetable, with wide range of vitamins and minerals. This review states very clearly the importance of baby spinach in terms of human health as it is loaded with nutrients 1which can prevent deficiency diseases and are essential for normal physiological function, and phytochemicals which are believed 1to help prevent chronic health problems such as cancer and heart disease, as well as other health problems associated with ageing. Both minerals and phytochemicals are essential to promote good health. Pre-harvest and post-harvest factors have effects on minerals and phytochemicals in vegetables. These factors may lead to lower or higher concentrations. (Berquist, 2006). According to Leskovar et al., 2009, the selection of suitable planting material, field preparation, fertilizer application, weeding and irrigation in absence of rain are some of the agronomic practices in cultivation of vegetables. Genetic factors, cultivar selection, harvesting and storage have influence in the Therefore, this present review serves as groundwork to a research to investigate and determine the effects of nitrogen, phosphorus & potassium fertilization, timing of application and method of application on bioactive compounds (antioxidants, flavonoids, total phenolic content & carotenoids) of baby spinach. The importance of baby spinach in human health can never be over emphasized; therefore it is crucial to investigate agronomic practices such as mineral nutrition (nitrogen, phosphorus & potassium fertilization) that can enhance the quality of baby spinach. This can contribute to South African food security and exports markets. CHAPTER 3 3. DESCRIPTION OF STUDY AREA AND RESEARCH METHODOLOGY 3.1 INTRODUCTION This section of thesis deals with the details of experiments which have been carried out. Chapter 3 provides a description of the study area, followed by a clarification of data collection methods and analyses techniques. The study employed quantitative research approach to address its objectives. 3.2 3MATERIALS AND METHODS 3. 2 .1 Experimental site The study was carried out at Agricultural Research Council - Vegetable and Ornamental Plant Institute (ARC-VOPI) in Roodeplaat farm, situated in the sourish mix of bushveld, 25 km 3north of central Pretoria, KwaMhlanga (R573) road; GPS coordinates: 25,56S;28,35E (Gauteng province, South Africa). The area is 4a relatively cool subtropical climate with summer rainfall and cold, dry winter. 3.2.2 Experimental site design and treatment details 3.2.2.1 Experimental site design 2Baby spinach (Spinacia oleracea L.)

cv. Ohio was used as a model crop in the studies reported in this thesis. Three trials were conducted to determine the optimum fertilizer rate(s). Seedbeds were prepared and filled with virgin red soil. A total of 12 seedbeds were prepared for all 3 trials. Each trial had 5 treatments, replicated 4 times. For each trial, 4 seedbeds were allocated and 20 plots were demarcated for planting. Each experimental plot size was 1.2x1m2. Timed sprinkler irrigation system was used for irrigation. All trails were planted twice in different times under a protected environment and planting of seedlings was done from February to March 2014 for first the 1st planting of trials and from April to June 2014 for the 2nd planting of trials. Spinach seeds were sown in seed trays filled with agro mix as a growing medium and later transplanted into beds. Thirty seedlings were transplanted into 3 rows on each plot with 20 cm between rows and 10 cm between plants. The 1st & 3rd rows were border plants, and the 2nd row was the data plants. Samples were collected only from the data plants as displayed below in Plate 1. Page | 29 Plate 31: A bed of baby spinach demarcated into 5 different plots and 5 different treatments of N, P and K as shown in Table 2 The spinach was grown and harvested after 35 to 45 days. The leaves were harvested manually using sharp scissors by simply cutting the leaves at the stem as demonstrated in Plate 2. Each trial was harvested twice at 35 days and 45 days as last harvest. 20Each harvested at 2 growth stages at 6-day intervals. The outer oldest leaves were harvested first and the centre of the plant was harvested as those leaves matured. This method was used to allow it to re-sprout and give another partial harvest. After harvest, the samples were labelled accordingly, weighed for fresh mass and oven dried for 48 hours at 35o C. Plate 2: Manually harvesting of baby spinach using sharp scissors 3.2.2.2 4Treatment details Three (N, P, and K) parallel trials were conducted under protected environment. Treatments consisted of 0, 45, 975, 105, 120 kg·ha- 1g N or P; and 0, 63, 85, 106, 127, 148 kg·ha-1 in 4a randomized complete block design (RCBD) with 5 treatments replicated 4 times as shown below in Table 1. 4Fertilizer sources applied were Limestone Ammonium Nitrate (LAN, N = 28%) for N trial, Superphosphate (P = 83%) for P trial and Potassium chloride (K = 50%) for K trial. N, P & K fertilizers were applied as side dressing applications in the form of granules at 3two weeks after sowing. Table 1: Treatment arrangement for Nitrogen (N), Phosphorus (P) and Potassium (K) trials N P K trial treatments Applied Nitrogen 3(kg/ha) Applied Phosphorus (kg/ha) Applied Potassium (kg/ha) 0 0 0 45 45 63 75 75 85 105 105 127 120 120 148 Z 22- 45N, P kg·ha- 1ha and 63 - 138K kg·ha-1 recommended rates in California, USA After the first experiment of N, P, K was conducted and completed as shown in Table 1, the results were analysed and the optimum rates of nitrogen, phosphorus, potassium were recorded. The maximum rates where the concentrations on bioactive compounds and trace elements in baby spinach increased were used to conduct the second experiment of this study which was NPK treatments combinations. The trial consisted of 5 treatments and replicated 4 times. The treatment combinations were arranged as follows: 0, 30:30:48; 45:45:63; 60:60:78; 75:75:93. Plate 3: Two plots of baby spinach displayed the variations in growth and leaves appearance in different NPK treatments 3.2.4 Chemical analyses of bioactive compounds After harvest, the leaf samples were collected in paper bags, labelled accordingly, weighed for fresh mass and oven dried for 48 hours at 350 C, and the compounds were extracted using suitable solvents. Dry weights of the plants were taken prior to grinding the leaf samples in a laboratory mill. After grinding, these samples were placed in a dry place until the chemical determinations could be made. 2**Analyses of the bioactive compounds were** conducted **using reversed-phase** high-performance liquid chromatography (HPLC). 3.2.4.1 Micro element content The minerals, that is, Mg, Fe, Zn & Se, were determined in a dilute solution of the ashed samples by atomic absorption spectrophotometer (3300 PerkinElmer) on 56**described in Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC),** edited by Horwitz, (2000). The results are expressed as dry weight basis. 3.2.4.2 Total phenolic content

4**About 15 g of finely ground leaf material was sieved.**4**Total polyphenol concentrations were determined using the Folin-Ciocalteu (Waterman and Mole, 1994) method. In this method, 0.5 ml of the filtrate extracts was added to 50-mL volumetric flasks and filled up to** 50mL **with deionized water. The contents were swirled to mix, and 0.5 ml of the solutions were pipetted and mixed into test tubes containing 2.5 ml of Folin- Ciocalteu phenol reagent (Fluka Ltd, Johannesburg, South Africa). Twenty (20) g of sodium carbonate was dissolved in 100 ml of distilled water, and 5 ml of sodium carbonate solution was added to the mixture in the test tubes. The mixture was shaken thoroughly, by inverting it several times, and allowed to stand for 2 h for completion of the reaction, when a blue** colour **was formed. Measurements were done at 760 nm using a spectrophotometer (Du 530 Cecil Instruments, Cambridge, UK). The standards (preparations of 0.05 g tannic acid) were dissolved in the extracting solvent (75% acetone) up to 50 ml. The standard serial dilutions of 1, 0.8, 0.6, 0.4, 0.2, 0, 0.08, 0.06, and 0.02 mg·mL–1 were prepared. The optical densities were converted into concentrations from a standard curve using 1 to 0.02 mg·mL–1 tannic acid with phenol reagent and sodium carbonate in a similar manner. The standard curve obtained had an** r2 **value of 0.987, passing through the origin.** 3.2.4.3 Total carotenoid content 4**About 15 g of finely ground leaf material was sieved.** For carotenoids gradient a reversed-phase HPLC elution was used, β-carotene for the carotenoids. 2**The detector used for quantification purposes and identification of carotenoids was a diode array detector.** Total carotenoid content content is described as mean ± standard deviation in mg 100 g–1 dry mass. 3.2.4.4Total antioxidant activity content 4About 15 g of finely ground leaf material was sieved. For antioxidants, 2TEAC (trolox equivalent antioxidant capacity, Miller et al., 1993) technique was used 14.TEAC is a Page | 34 spectrophotometric technique that measures the relative ability of hydrogen-donating antioxidants to scavenge the ABTS+ radical cationchromogen in relation to that of Trolox, the water soluble vitamin E analogue which is used as an antioxidant standard. The ABTS+ was produced by mixing equal volume of 8mM ABTS with 3mM potassium persulfates was prepared in distilled water and allow to react in the dark for at least 12 hours at room temperature before use. The ABTS+ solution was diluted with a phosphate buffer solution (pH 7.4) mixed with 0.2 M of NaH2PO4, 0.2 M NaHPO4 and 150mM 14NaCl in 1 litre of distilled water, with pH adjustment using NaOH when necessary. A freshly-prepared solution was used for each analysis. The ABTS+ solution (2900µl) was added to the methanol extracts of tea (100 µl) of Trolox in a test tube and mixed. Absorbencies reading (at 734nm) were14taken after 30 minutes (for the samples) and 15 minutes (for the standard) of the initial mixing of the samples and standard, respectively. The results were expressed as µM Trolox equivalents /g of sample on dry weight basis. 3. 2.4.5 Total flavonoid content content 4About 15 g of finely ground leaf material was sieved.16Flavonoids were analyzed by reversed phase HPLC, as described by Bergquist et al., 2005. For flavonoids gradient a reversed-phase HPLC elution was used. 20Flavonoids were identified using LC-MS/MS (liquid chromatography combined with mass spectrometry). 16Samples were extracted in triplicate under dim green light, using 60mg plant material with 5mL 40% methanol. Before choice of solvent, different compositions of the extraction solution (40, 50 and 85% methanol or ethanol in water) were tested. The16samples were shaken for 20 h at 4 ◦C, 150 rpm and centrifuged at 10 000 × g at 4 ◦C for 10 min. The supernatants were transferred to glass vials and stored at −80 ◦C until analyzed by HPLC. Flavonoids16concentration was quantified at 340nm using an external spiraeoside standard (Extrasynthese, Lyon, France). The flavonoids were identified by comparing retention times and absorption spectra with compounds previously identified by LC–MS/MS. 3.2.4.6 Vitamin C Ascorbic acid content was determined according to, "Association of Official Analytical Chemists (AOAC). Ascorbic acid content was expressed as mg/100 g, dry weigh basis. 3.2.3.7 Method for N (nitrogen) determination The sample is used directly (in finely milled or powder form) for N determinations on a

Carlo Erba NA 1500 C/N/S Analyzer, using approximately 8 to 14 mg sample weighed into a tin foil container for each determination. (Adapted from Ref. 1 to 2). The method is a dry oxidation method generally known as the Dumas method. The sample and tin container are ignited at high temperature (1020 oC) in oxygen (on a chrome oxide catalyst) to produce carbon dioxide, nitrogen gas and oxides of nitrogen (plus other oxides etc.). The gases produced pass through silvered cobalt oxide, then a column of copper (540 oC), which reduces the oxides of nitrogen to nitrogen gas (and removes excess free O2). After removal of water vapour and CO2 by traps, the N2 gas is finally separated from any traces of other gases by gas chromatography using a helium carrier gas and detected by a thermal conductivity detector. The instrument is calibrated against a pure organic compound of known composition. The compound chosen for our calibration standard is the ethyl ester of 4-Aminobenzoic acid, which contains 8,48% N. PeakNet software (Dionex Corporation, May 1998), with an external A/D interface (UI20 Universal Interface, Dionex) is used for data collection, peak integration, calibration and computation of concentrations (Ref. 3). 3.2.3.8 Sample Extraction for Nitrate (and Nitrite) A sub-samples of the sample was extracted with distilled water, using 0.2g sample to 50ml water and shaken on a mechanical shaker for 30 minutes, before filtering. For the N Control sample, only 0.096g was used instead of 0.2g, as there was insufficient sample available. 3.2.3.9 Determination of Nitrate (and Nitrite) The water extract solution was analysed by ion chromatography, which detects nitrate as well as most of the other major anions (nitrite, chloride, fluoride, sulphate etc.), by separating the anions on an ion exchange column and detecting them with a conductivity detector. The nitrite was below the detection limit for all samples. The nitrate N was calculated from the N using a factor of 0.226, which is the mass of a N atom divided by the mass of NO3 (a N atom plus 3 O atoms). 3.2.3.10 Computation of protein N and protein The protein N was estimated by subtraction of the nitrate N from the total N. This estimate is expected to be slightly too high, because chlorophyll N, ammonium N and any other possible forms of non-protein N (other than nitrate) have not been excluded. While values for chlorophyll were available, these were on a mass/ unit area basis, not a mass per mass basis, so without the thickness and density of the leaves, they could not be converted to a mass per unit mass basis. From the estimate of non-protein N, the protein was estimated by using a factor of 6.25. This is the default factor usually used for conversion of N to protein when the correct factor for the product of interest is unknown. It is based on the assumption that the protein contains 16.0% N. However, this factor of 6.25 is most appropriate for meat products. Many types of plant protein have a higher percentage of N than 16.0%, so that the best protein factor for many plant products is slightly less than 6.25 and for some plant types slightly below 6.0. Thus, the estimate of the protein content may be slightly too high for two reasons. Firstly, not all the non-protein N has been excluded, and secondly the protein factor of 6.25 might be slightly too high. 3.2.3.11 Method for Perchloric + Nitric Acid Sample Digestion: 1g of sample is digested with 7ml HNO3 (conc. nitric acid) and 3ml HClO4 (perchloric acid) at temperatures up to 200°C and brought to volume in a 100ml vol. flask. (Adapted from Ref. 4). 3.2.3.12 ICP-OES Determination of P & K An aliquot of the digest solution is used for the ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometric) determination of P & K. The ICP-OES is a multi-element instrument. The instrument used (Varian Liberty Series II) is a Page | 37 sequential instrument, where the elements are determined almost simultaneously, with only a few seconds between each element. Each element is measured at an appropriate emission wavelength, chosen for high sensitivity and lack of spectral interferences. The wavelengths used were: - P: 213.618nm and K: 769.896nm. The instrument is set up and operated according to the recommended procedures in the instrument manual. It is calibrated against a series of standard solutions, containing all the elements of interest in the proportions found in typical leaf samples. (Unpublished method developed by Mike Philpott at ARC-ISCW, based on the recommended procedures in the instrument manual: Ref. 5). 3.2.4 Data collection For all the treatments, at harvest (31 March 2014; 7, 11, 18 April 2014; 9, 23 May 2014; 27 June 2014 and 28 July 2014), fresh and dry shoot mass (g) weighed by Symmetry PR Precision Scale recorded. Parameters recorded were total phenolic content, total antioxidant activity , total carotenoid content, total flavonoid content, vitamin C, magnesium, iron, zinc, selenium, total leaf tissue nitrogen, phosphorus, and potassium. 3.2.5 4**Statistical analyses. Data** collected, **were subjected to analysis of variance (ANOVA) using the GLM (general linear model) procedure of SAS, version 8.0. (SAS Institute, 1999). In all trials, treatment sums of squares were partitioned into linear and quadratic polynomial contrasts for total phenolic content**, **total** antioxidants activity, total carotenoid content, total flavonoid content, vitamin C, magnesium, iron, zinc, selenium, total leaf tissue nitrogen, phosphorus, and potassium. CHAPTER 4 4. RESEARCH RESULTS AND DISCUSSION 4.1 RESEARCH RESULTS This results section presents the findings of the study in relation to the research objectives based on the collected and analysed data. In these experiments, chemical composition analysis was done for trace elements (Magnesium (Mg), Iron (Fe), Zinc (Zn) & Selenium (Se) and bioactive compounds (total phenolic content, total carotenoid content, total flavonoid content, total antioxidant activity and Vitamin C). The results show the variations on bioactive compounds of baby spinach in relation to different rates of applied 8nitrogen (N), phosphorus (P) and potassium (K) fertilizers as well as the NPK treatment combinations. 4.1.1 Response of chemical composition of baby spinach to nitrogen nutrition Table 3 indicates the variations on total phenolic content, total antioxidant activity, total carotenoid content & vitamin C, magnesium, iron, zinc and selenium concentrations in baby spinach as a result of nitrogen applied at different rates. Table 3: Response of chemical concentrations in baby spinach to different levels of nitrogen (N) nutrition (dry weight basis) Nitrogen Total Total Total Applied Magnesium Iron Zinc Selenium Phenols Carotenoids Flavonoids (kg·ha-1) (ppm) (ppm) (ppm) (ppm) 31(mg·g-1) (mg·g-1) (mg·g-1) Control 1. 01a 0.91a 1. 123a 0. 001a 3.07b 0.89a 4.0b 45 1.01a 0.11a 1.01a 0.001a 8.1a 1.01a 8.1a 75 1.01a 0.91a 1.123a 0.001a 6.21a 1.5a 7.21a 85 1.01a 0.01a 0.01b 0.001a 6.1a 1.48a 7.1a 105 1.01a 0.91a 1.123a 0.001a 6.01a 1.39a 6.01a 120 1.02a 0.89a 1.021a 0.001a 5.9b 1.39a 6.01a Significance NS NS NS NS Q NS Q Total antioxidant activity Vitamin Activity C (mg·g-1) (mg·g-1) 0.49b 4.46b 3.01a 10.9a 2.13a 8.12a 2.01a 7.12a 2a 6.15a 1.9b 6.1b Q Q Protein N % 1.34b 4.01a 4.26a 4.22a 4.22a 4.20a Q Total N% 1.34b 4.01a 4.31a 4.34a 4.54a 4.60a Q Nitrate % 90.02b 0.04b 0.23b 0.57b 1.33a 1.40a Q Leaf Protein %* 98.3b 25.0a 26.6a 26.3a 26.5a 26.2a Q Linear (L) 3or quadratic (Q) effects significant at P = 0.05 (*), 0.01 (**) or non-significant (NS) Means 38with the same letter are not significantly different at 5% level of probability Page | 40 4.1.1.1 Response of trace elements (Magnesium, Iron, Zinc and Selenium) to nitrogen nutrition Table 3 3showed that different nitrogen levels did not have any significant effect on magnesium, iron, zinc and selenium) concentrations of baby spinach leaves. 4.1.1.2 Response of total phenolic content to nitrogen nutrition Results in Table 3 and Figure 3.1 show that Total phenolic content increased quadratically in response to nitrogen application. Total phenolic content level peaked at 45kg·ha-1. The application of 45kg·ha-1 improved the total polyphenol content reaching maximum at 8.1mgg-1 (Table 3 & Figure 3.1). The total phenolic content increased from 0 to 45kg·ha-1. The results showed that the total phenolic content of baby spinach deteriorated with increasing nitrogenous fertilizer rates ranges from 75 to120 kg·ha-1. The difference between the highest (8.1 mg·g-1) and lowest (3.07 mg·g-1) mean value on Total phenolic content was 5.03 mg·g-1 on dry mass basis (Table 3 & Figure 3.1). 10 9 Total phenolic content (mg·g-1) 8 7 6 5 4 3 2 1 0 Control 45 75 85 105 120 N Kg·ha-1 Figure 3.1: Total phenolic content concentrations of baby spinach at different rates of nitrogen application (dry weight basis) 4.1.1.3 Response of total carotenoid content to nitrogen nutrition The 3results showed that different nitrogen levels applied did not have any significant effect on the carotenoids content of baby spinach leaves (Table 3). This suggests that there was no significant increase observed on the concentration of carotenoids regardless of the level of N applied. 4.1.1.4 Response of total flavonoid content to nitrogen nutrition Concentration of total flavonoid content showed variations in baby spinach leaves (Table 3 & Figure 3.2). The lowest total flavonoid content in the leaves concentration were 4.0 mg 19·g-1 and the highest concentration was 8 .1 mg·g-1 (Table 3 & Figure 3.2). All treatments significantly improved total flavonoid content content. The significant increase ranges from

824.0 mg·g-1 to 8.1 mg·g-1 after treatments application. The difference between the lowest and the highest concentration of total flavonoid content in baby spinach leaves was 4.1 mg·g-1 on dry mass basis. 10 9 Total flavonoid content (mg·g-1) 8 7 6 5 4 3 2 1 0 Control 45 75 85 105 120 N Kg·ha-1 Figure 3.2: Total flavonoid content concentrations of baby spinach at different rates of nitrogen application (dry weight basis) 4.1.1.5 Response of total antioxidant activity to nitrogen nutrition The application of 45kg·ha-1 improved the total antioxidant activity content reaching 3.01 mg·g-1 (Table 3 & Figure 3.3). The total antioxidant activity content of 7plants under 45 kg·ha-1 was significantly higher compared to the control (0 kg·ha-1). The significant increase ranges from 0.49 mg·g-1 to 3.01 mg·g-1 after treatments application. The difference between the highest and lowest mean value on total antioxidant activity content was 3.52 mg·g-1 on dry mass basis (Table 3 & Figure 3.3). 4 Total antioxidant activity (mg·g-1) 3.5 3 2.5 2 1.5 1 0.5 0 Control 45 75 85 105 120 N Kg·ha-1 Figure 3.3: Total antioxidant activity concentrations of baby spinach at different rates of nitrogen application (dry weight basis) 4.1.1.6 Response of Vitamin C to nitrogen nutrition Results in Table 3 & Figure 3.4 showed that there was a variation in concentrations of Vitamin C in baby spinach. Vitamin C was quadratically increased by application. Highest concentrations were at 45 kg·ha-1. Vitamin C 4response to N occurred between 0 to 45 kg·ha-1. The lowest vitamin C in the leaves concentrations was 4.46 mg·g-1 and the highest concentrations was 10.9 mg·g-1 (Table 3 & Figure 3.4). The difference between the highest and lowest mean value on total antioxidant activity content was 6.44 mg·g-1 on dry mass basis (Table 3 & Figure 3.4). 14 12 Vitamin C (mg·g-1) 10 8 6 4 2 0 Control 45 75 85 105 120 N Kg·ha-1 Figure 3.4: Vitamin C concentrations of baby spinach at different rates of nitrogen application (dry weight basis) 4.1.7 Percentage protein N Percentage protein nitrogen quadratically increased with increasing nitrogen ranging from 1.34% to 4.26% (Table 3 & Figure 3.5). Nitrogen application 3significantly increase % protein N of baby spinach compared to control reaching maximum at 945kg·ha- 1. The difference between the highest and lowest value is 2. 92% (Table 3 & Figure 3.5). 5 4.5 4 %Leaf tissue N 3.5 3 2.5 2 1.5 1 0.5 0 Control 45 75 85 105 120 N Kg·ha-1 Figure 3.5: Leaf tissue nitrogen content in baby spinach 4.1.1.8 Percentage total N % The highest percentage total nitrogen of 4.60% was observed at 120 kg·ha-1, 3causing a linear response with high rates of nitrogen applied. The difference between the highest value and lowest value is 3. 26 (Table 3 & figure 3.6). 6 5 4 Total N% 3 2 1 0 Control 45 75 85 105 120 N Kg·ha-1 Figure 3.6: Total N% in baby spinach 4.1.1.9 Percentage Nitrate The results in Table 3 3showed that there is a connection between nitrogen applied and percentage leaf nitrate found in the leaves of baby spinach. Nitrate percentage in the 9baby spinach leaves increased with increasing nitrogen application reaching maximum of 1.40% at 120 kg·ha- 1 (Table 3 3).The difference between the highest and lowest value is 1.309% (Table 3). 4.1.2 Response of chemical composition and trace elements to phosphorus nutrition Table 4 below indicates the variations on total phenolic content, total antioxidant activity, total carotenoid content & vitamin C, magnesium, iron, zinc and selenium in baby spinach as a result of phosphorus applied at different rates. Table 4: Response of chemical concentrations in baby spinach to different levels of phosphorus (P) nutrition (dry weight basis) Phosphorus Total Total Total Applied Magnesium Iron Zinc Selenium Phenols Carotenoids Flavonoids (kg·ha-1) (ppm) (ppm)

(ppm) (ppm) 31(mg·g-1) (mg·g-1) (mg·g-1) Control 1. 01a 0.91a 1. 123a 0. 221a 3.13b 0.89a 5.13b 45 1.01a 0.11a

1.01a 0.201a 8.10 a 1.01a 9.32a 75 1.01a 0.81a 1.123a 0.211a 7.21a 1.60a 7.31a 85 1.01a 0.21a 0.01a 0.201a 5.10b 1.58a 7.41a 105 1.01a 0.91a 1.123a 0.221a 4.01b 1.49a 6.21b 120 1.01a 0.002a 1.021a 0.201a 4.90b 1.39a 6.01b Significance NS NS NS NS Q Q Total antioxidant activity Vitamin Activity C (mg·g-1) (mg·g-1) 3.5b 5.42b 4.01a 9.9a 2.93b 8.12a 2.81b 7.32a 2.5b 6.55b 2.4b 6.4b Q Q % Leaf tissue P 0.526b 0.968a 0.948a 0.950a 1.115a 1.119a Q Protein %* 13.3c 21.3b 26.4a 26.3a

26.8a 26.9a Q Linear (L) 3or quadratic (Q) effects significant at P = 0.05 (*), 0.01 (**) or non-significant (NS) Means

38with the same letter are not significantly different at 5% level of probability Page | 47 4.1.2.1 Response of trace

elements to phosphorus nutrition Results in Table 4 showed that regardless of different levels of phosphorus applied, there was no significant effect on magnesium, iron, zinc and selenium concentrations of baby spinach. 4.1.2.2 Response of total phenolic content to phosphorus nutrition Results in Table 4 & Figure 4.1 show that there was a quadratic increase of total phenolic content with application of phosphorus nutrition. In the P trial, total phenolic content reached their maximum at 45 kg·ha-1 with total

phenolic content concentration of 8.10 mg·g-1. 4Most of total phenolic content response to P occurred between 0 to

75 kg·ha-1. The difference between the highest (8.10 mg·g-1) and lowest (3.13 mg·g-1) mean value on Total phenolic

content was 4.97 mg·g-1 on dry mass basis (Table 4 & Figure 4.1). Total phenolic content concentration mg·g-1) 9 8 7 6 5 4 3 2 1 0 Control 45 75 85 105 120 P kg·ha-1 Figure 4.1: Total phenolic content concentrations of baby spinach at different rates of phosphorus application (dry weight basis) 4.1.2.3 Response of total carotenoid content to phosphorus nutrition Different levels of

phosphorus 9applied did not have any significant effect on the total carotenoid content in baby spinach leaves with

the lowest value of 0.89 mg·g-1 and the highest value of 1.60 mg·g-1 (Table 2). Thus, there was no significant increase on the concentration of carotenoids regardless of the levels of P applied. 4.1.2.4 Response of total flavonoid content content to phosphorus nutrition The results showed that the total flavonoid content concentration was quadratically increased by

phosphorus nutrition. Highest concentrations were observed at 45 kg·ha-1 (Table 3 & Figure 4.2). 4Most of the total

flavonoid content response to P occurred between 0 to 45 kg·ha-1. The highest total concentration of total flavonoid

content was 9.32 mg·g-1and the lowest mean value was 5.13 mg·g-1. 3The difference between the highest mean

value and the lowest mean value is 4.19 mg·g-1 (Table 3 & Figure 4.2). 12 Total flavonoid content (mg·g-1) 10 8 6 4 2 0

Control 45 75 85 105 120 P Kg·ha-1 Figure 4.2: Total flavonoid content concentrations of baby spinach at different rates of phosphorus application (dry weight basis) 4.1.2.5 Response of total antioxidant activity of phosphorus nutrition In the P trial, total antioxidant activity concentration reached their maximum at 45 kg·ha-1 with total antioxidant activity concentration of 4.01 mg·g-

1 (table 4 & Figure 4.3). Most of total antioxidant activity phenols 4response to P occurred between 0 to 45 kg·ha-1.

The difference between the highest (4.01 mg·g-1) and lowest (3.50 mg·g- 1) mean value on Total phenolic content was 0.5 mg·g-1 on dry mass basis (Table 4 & Figure 4.3). Total antioxidant activity (mg·g-1) 4.5 4 3.5 3 2.5 2 1.5 1 0.5 0 Control 45 75 85 105 120 P Kg·ha-1 Figure 4.3: Total antioxidant activity concentrations of baby spinach at different rates of phosphorus application (dry weight basis) 4.1.2.6 Response of Vitamin C to phosphorus nutrition Results in Table 4 & Figure 4.4 showed a variation in concentrations of Vitamin C in baby spinach. There was a quadratically increased in Vitamin C due to phosphorus nutrition.

Highest concentrations were at 45 kg·ha-1. Vitamin C 4**response to N occurred between 0** to 45 **kg·ha-1.** The lowest

concentration **in** Vitamin C in the leaves of baby spinach was 5.42 mg·g-1 and the highest concentrations was 9.9 mg·g-1.

The difference between the highest and lowest mean value on total antioxidant activity content was 4.48 mg·g-1 on dry mass

(Table 4 & Figure 4.4). 12 10 72**Vitamin C (mg**· g-1) **8 6 4 2 0** Control 45 75 85 105 120 P Kg·ha-1 Figure 4.4: Vitamin C

concentrations of baby spinach at different rates of phosphorus application (dry weight basis) 4.2.2.7 Percentage leaf tissue P Percentage leaf tissue phosphorus was quadratically increased and ranged from 0.526% to 0.968 % (Table 4 & Figure 4.5). Phosphorus applied significantly increase % leaf tissue P of baby spinach compared to control reaching maximum at

9**45kg·ha-** 1. **The difference between the highest and lowest value is** 0.442% **(Table** 3 & Figure 4.5). 1.4 1.2 % Leaf

tissue P 1 0.8 0.6 0.4 0.2 0 Control 45 75 85 105 120 P Kg·ha-1 Figure 4.5 Percentage leaf tissue N in baby spinach 4.2.2.8

3**Protein %* Protein percentage seemed to have increased at 75kg·ha-** 1 **which showed that there is a relationship between the nitrogen**

applied and protein found in the leaves of baby spinach. Increasing the rates of nitrogen applied to 120kg·ha- 1 **did not** exhibit

3**protein content. The difference between the highest value and lowest value is 18.3%* (Table** 4 & Figure 4.6). 35.00 30.00 25.00

Protein %* 20.00 15.00 Protein %* 10.00 5.00 0.00 Control 45 75 85 105 120 P Kg·ha-1 Figure 4.6 Protein %* in baby spinach 4.1.3 Response of chemical composition and trace elements to potassium nutrition Table 5 below indicates the variations on total phenolic content, total antioxidant activity, total carotenoid content & vitamin C, magnesium, iron, zinc and selenium in baby spinach as a result of potassium applied at different rates. Table 5: Response of chemical concentrations in baby spinach to different levels of potassium (K) nutrition (dry weight basis) Potassium Applied Magnesium Iron (kg·ha-1) (ppm) (ppm) Control 1.23a 0.81a 63 1.11a 0.11a 85 1.12a 0.81a 106 1.11a 0.21a Total Total Total Zinc Selenium Phenols Carotenoids Flavonoids

(ppm) (ppm) 31**(mg·g-1) (mg·g-1) (mg·g-1)** 1.123a **0.** 221a 1.01b 0.89a 3.01b 1.01a 0.201a 3.13b 1.89a 7.13a 1.123a

0.211a 7.10a 1.71a 7.10a 1.01a 0.201a 6.21a 1.50a 6.21a 127 1.11a 0.91a 1.123a 0.221a 5.10a 1.48a 148 1.12a 0.82a 1.021a 0.201a 5.01a 1.39a Significance Ns Ns Ns Ns Q Q Total antioxidant activity (mg·g-1) 0.49b 0.50b 5.01a 3.13a 6.10a 2.01a 6.01a 2.23a Q Q Vitamin (mg·g-1) C 3.51b 4.42b 7.90a 6.12a 5.12a 5.15a Q % Leaf tissue K 4.61c 6.25b 6.63b 7.60a 7.49a

7.40a Q Protein %* 10.8b 14.3a 14.4a 11.1b 11.4b 11.5b Q Linear (L) 3**or quadratic (Q) effects significant at P = 0.05**

(*), 0.01 (**) or non-significant (NS) Means 38**with the same letter are not significantly different at** 5% level of

probability Page | 53 4.1.3.1 Response of trace elements in baby spinach in potassium nutrition Results in Table 5 showed that regardless of different levels of potassium applied, there was no significant effect on the trace elements magnesium, iron, zinc and selenium concentrations of baby spinach. 4.1.3.2 Response of total phenolic content to potassium nutrition The results in Table 5 & Figure 5.1 indicate that the total phenolic content concentration increased quadratically in response to potassium application. Total phenolic content concentration level peaked at 85kg·ha-1. The application of 85kg·ha-1 improved the total polyphenol content reaching 7.10 mg·g-1 (Table 5 & Figure 5.1). 4Most of the total phenolic content response to K occurred between 0 to 85 kg·ha-1. The difference between the highest (7.10 50mg·g-1) and lowest (1. 01 mg·g-1) mean value on Total phenolic content was 6.09 mg·g-1 on dry mass basis (Table 5 & Figure 5.1). 9 Total phenolic content (mg·g-1) 8 7 6 5 4 3 2 1 0 63 85 106 127 148 K Kg·ha-1 Figure 5.1: Response to various levels of potassium fertilizer on total phenolic content concentrations in baby spinach (dry weight basis) 4.2.4.3 Response of total carotenoid content to potassium nutrition Different levels of 9potassium applied did not have any significant effect on the total carotenoid content in baby spinach leaves. In K trial, there was no significant increase on the concentration of carotenoids regardless of the levels of K applied (Table 5). 4.2.4.4 Response of total flavonoid content to potassium nutrition The results showed that the total flavonoid content concentration quadratically increased by potassium nutrition. Highest concentrations were observed at 63 kg·ha-1 (Table 5). 4Most of the total flavonoid content response to K occurred between 0 to 85 kg·ha-1. The highest total concentration of total flavonoid content was 7.13 mg·g-1and the lowest mean value was 3.01 mg·g-1 (Table 5). The difference 79between the lowest and the highest mean values was in with the difference of 4.12 mg·g-1. 4.2.4.5 Response of total antioxidant activity to potassium nutrition The total antioxidant activity content peaked at 85 kg·ha-1 reaching a maximum of 5.01 mg·g-1 (Table 5 & Figure 5.2). The highest mean value was 5.01 mg·g-1 and the lowest was 0.49 mg·g-1. The difference between the highest and lowest mean value on total antioxidant activity content was 4.52 mg·g-1 on dry mass basis (Table 5 & Figure 5.2). 7 Total antioxidant activity (mg·g-1) 6 5 4 3 2 1 0 63 85 106 127 148 -1 K Kg·ha-1 Figure 5.2: Response to various levels of potassium fertilizer on total antioxidant activity concentrations in baby spinach (dry weight basis) 4.2.4.6 Response of Vitamin C to potassium nutrition Results in Table 5 indicted that there was a variation in concentrations of Vitamin C in baby spinach. Vitamin C was quadratically increased by nutrition. Highest concentrations were at 85 kg·ha-1. Vitamin C 4response to N occurred between 0 to 85 kg·ha-1. The lowest vitamin C in the leaves concentrations was 3.51 mg·g-1 and the highest concentrations was 7.90 mg·g-1 (Table 5 & Figure 5.3). The difference between the highest and lowest mean value on total antioxidant activity content was 4.39 mg·g-1 on dry mass basis (Table 5 & Figure 5.3). 9 8 7 Vitamin C (mg·g-1) 6 5 4 3 2 1 0 63 85 106 127 148 K Kg.ha-1 Figure 5.3: Response to various levels of potassium fertilizer on Vitamin C concentrations in baby spinach (dry weight basis) 4.2.2.7 % Leaf tissue K Potassium 3applied significantly increase % protein content (value) of baby spinach compared to control reaching maximum at 945kg·ha- 1. The difference between the highest and lowest value is 2.881% (Table 5 & Figure 5.4). 9.00 8.00 7.00 % Leaf tissue 6.00 5.00 4.00 3.00 2.00 1.00 0.00 Control 63 85 106 127 148 K Kg·ha-1 Figure 5.4: % Leaf tissue K in baby spinach 4.2.2.9 3Protein %* Protein percentage seemed to have increased at 63 kg ·ha- 1 which showed that there is a relationship between the nitrogen applied and protein found in the leaves of baby spinach. Increasing the rates of nitrogen applied to 148 kg ·ha- 1 did not exhibit 3protein content. The difference between the highest value and lowest value is 18.3% (Table 5 & Figure 5.5). Protein %* 16.00 14.00 12.00 10.00 8.00 6.00 4.00 2.00 0.00 Control 63 85 106 127 148 K Kg·ha-1 Figure 5.5: Protein%* in baby spinach 4.1 8.4 Effect of combinations of different rates of nitrogen, phosphorus & potassium (NPK) fertilizers on chemical concentrations in baby spinach

Table 6 below indicates the variations on total phenolic content, total carotenoid content, total antioxidant activity , total flavonoid content, vitamin C, magnesium, iron, zinc and selenium in baby spinach as a result of combinations of different rates of nitrogen, phosphorus & potassium fertilizers applied. Table 6: Response of chemical composition in baby spinach to different 8levels of nitrogen (N), phosphorus (P), and potassium (K) treatment combinations nutrition (dry mass basis)

Nitrogen/ Phosphorus /Potassium Applied (kg·ha-1) Control 30:30:45 45:45:60 75:75:90 60:60:75 Magnesium (ppm) 1.23a 1.11a 1.12a 1.11a 1.11a Iron (ppm) 0.81a 0.11a 0.81a 0.21a 0.91a Total Total Total Total antioxidant activity Vitamin Zinc Selenium Phenols Carotenoids Flavonoids Activity (ppm) (ppm) 31(mg·g-1) (mg·g-1) (mg·g-1) (mg·g-1) (mg·g-1) C 1. 123a 0.221a 1. 01b 0. 89b 3.01b 0.49b 3.51a 1.01a 0.201a 5.01a 1.89a 3.01b 4.49a 3.51a 1.123a 0.211a 6.13a 3.89a 7.13a 6.50a 4.42a 1.01a 0.201a 5.10a 1.71a 5.10a 3.01a 3.91a 1.123a 0.221a 4.21a 1.50a 4.21a 2.13b 2.12a 75:75:90 1.12a 0.82a 1.021a 0.201a 4.10a 1.48a 3.10a 2.01b 2.10a Significance 3Ns Ns Ns Ns Q Q Q Q Ns Linear (L) or quadratic (Q) effects significant at P = 0.05 (*), 0.01 (**) or non-significant (NS) Means 38with the same letter are not significantly different at 5% level of probability Protein %* 20.0b 27.8a 25.4a 26.5a 27.1a 27.3a Q %N %P 3.21b 0.19a 4.51a 0.45a 4.10a 0.49a 4.30a 0.60a 4.42a 0.53a 4.47a 0.50a Q Ns %K 3.45b 5.90a 5.79a 5.86a 6.13a 6.19a Q Page | 59

4.2.5.1 Response of trace elements in baby spinach to NPK nutrition The treatment combinations of NPK 3rates applied on baby spinach did not show any significance effect on the magnesium, iron, zinc and selenium concentrations (Table 6). Therefore, based on Table 6 there were no significant differences observed on trace elements regardless of different treatment combinations of NPK applied (Table 6). 4.2.5.2 Response of total phenolic content to NPK nutrition The treatments of N45, P45, K60 kg·ha-1 improved the concentrations of total phenolic content (Tables 6 & Figure 6.1). This is in agreement with the treatment combination of NPK (45:45:60) which indicated a significantly increased of total phenolic content in baby spinach compared to the control. The mean value of total phenolic content at NPK combination (45:45:60) was 6.13 mg·g-1 higher than all other treatments (Table 6 & Figure 6.1). 8 7 Total phenolic content (mg·g-1) 6 5 4 3 2 1 0 Control 30:30:48 45:45:60 75:75:90 60:60:75 75:75:90 NPK Kg·ha-1 Figure 6.1: Response to various levels of NPK fertilizers on total phenolic content concentrations in baby spinach (dry weight basis) 4.2.5.3 Response of total carotenoid content to NPK nutrition Total flavonoid content 7content was not significantly affected by treatment combinations of NPK fertilizers, although the plants under N45, P45, K60 kg·ha-1 had the highest total flavonoid content content of 3.89 mg·g-1 compared to other treatment combinations including Page | 60 the control (Table 6 & Figure 6.2). 7There was no significant difference in total carotenoid content among other treatment combinations of NPK.9The difference between the highest mean value and the lowest mean value was 3 mg ·g- 1. 5 4.5 Total carotenoid content (mg·g-1) 4 3.5 3 2.5 2 1.5 1 0.5 0 Control 30:30:48 45:45:60 75:75:90 60:60:75 75:75:90 NPK Kg·ha-1 Figure 6.2: Response to various treatment combinations of NPK fertilizers on total carotenoid content concentrations in baby spinach (dry weight basis) 4.2.5.4 Response to total flavonoid content content to NPK nutrition Total flavonoid content 7content was significantly affected by NPK treatment combinations. The plants under N45, P45, K60 kg·ha-1 had the highest total flavonoid content content of 7.13 mg·g-1 compared to the control and other treatment combinations (Table 6 & Figure 6.3). 7There was no significant difference in total flavonoid content content among other treatment combinations of NPK and 80a slight decrease in the total flavonoid content content was observed with the maximum treatment combinations. 9 Total flavonoid content (mg·g-1) 8 7 6 5 4 3 2 1 0 Control 30:30:48 45:45:60 75:75:90 60:60:75 75:75:90 NPK Kg·ha-1 Figure 6.3: Response to various treatment combinations of NPK fertilizers on total flavonoid content concentrations in baby spinach (dry weight basis) 4.2.5.3 Response of total antioxidant activity content to NPK 7The effect of NPK on the anti-oxidant activities and components of baby spinach leaves is presented in (Table 6 & 6.4). The plants under N45, P45, K60 kg·ha-1 had the highest antioxidant activity of 6.5 mg·g-1 compared to other treatment combinations including the 57control (0 kg·ha-1). The Total phenolic content of plants under N45, P45, K60 kg 7·ha-1 was significantly higher than the plants under other NPK levels (Table 6 & Figure 6.4). Total Antioxidant Activity (mg·g-1) 8 7 6 5 4 3 2 1 0 -1 Control 30:30:48

45:45:60 75:75:90 60:60:75 75:75:90 NPK Kg·ha-1 Figure 6.4: Response to various levels of NPK fertilizers on total antioxidant activity concentrations in baby spinach (dry weight basis) 4.2.5.6 Response of Vitamin C content to NPK nutrition The results showed that NPK treatment combinations 3applied on baby spinach did not show any significance influence on the Vitamin C content. 4.2.5.7 Leaf protein content (%) The treatment combinations of N30, P30 and K48 (kg·ha-1) increased leaf protein content percentage ranging from 20 to 27.8% (Table 6). Treatment combinations of N30, P30, K48 kg·ha-1 significantly improved leaf protein content percentage (Table 6). 4.2.5.8 Percentage leaf tissue N Percentage leaf tissue potassium was increased and ranged from 3.21 to 4.51% (Table 6). The treatment combinations of N30, P30, K48 (kg·ha-1) improved the concentrations of leaf tissue N (%) (Table 6) 4.2.5.9 Percentage leaf tissue P All treatment combinations of NPK rates applied on baby spinach did not show any significance effect on percentage leaf tissue (Table 6) 4.2.5.10 Percentage leaf tissue K Percentage leaf tissue potassium was increased and ranging from 3.45 to 6.19% (Table 6). 4.3 DISCUSSION 4.3 77.1 Introduction This section presents the findings of the study comparing with other related conducted studies, looking at the similarities and the contrasts in the findings. Marketable baby spinach 8production requires suitable levels of nitrogen (N), phosphorus (P), and potassium (K) to provide high-quality postharvest qualities required for longer shelf life and health benefits. Therefore, the section deliberates on the outcomes of the study in an effort to consolidate and recommend the adequate NPK fertilizer rates or levels suitable for South African baby spinach production. The following findings discussed in this section: the effect of different rates of N, P & K fertilizers and the NPK treatment combinations on chemical concentrations (total phenolic content, total carotenoid content, total flavonoid content and total antioxidant activity) and trace elements (Mg, Fe, Zinc & Se) in baby spinach. 4.3.2 The response of chemical concentrations and trace elements to nitrogen nutrition in baby spinach The parameters recorded in this study included total phenolic content, total carotenoid content, total antioxidant activity , total flavonoid content, vitamin C, magnesium, iron, zinc and selenium. Table 3 and Figures 3.1 to 3.6 showed the variation in chemical concentrations in the leaves of baby spinach as affected by applied different levels of nitrogen nutrition. The results showed that the application of nitrogen at different levels did not have any significant effect on the trace elements (Magnesium, Iron, Zinc & Selenium) concentrations of baby spinach leaves (Table 3). These nutrients are present in smaller quantities in baby spinach, excluding iron which is regarded as one of the major nutrients in baby spinach (Hedges &Lister, 2007). On the contrary to the results of this study, Lefsrud et al, 2007 reported magnesium and zinc responded to nitrogen treatments, Zinc decreased as nitrogen level increases while Magnesium increases in response to increase in nitrogen treatment level. Other findings by 24Wang et al., (2008) are that nitrogen fertilization increases the ratio of acids and sugars and reduces of calcium, magnesium and soluble sugars. Studies by Chenard et al., 2005 reported that leaf iron, manganese and molybdenum decreased with increases in nitrogen in nutrient solutions. Increasing solution nitrogen resulted in quadratic increases in leaf calcium, magnesium, sulfur & boron Chenard et al., 2005. In this study the results showed that Total phenolic content in baby spinach increased quadratically in response to different levels of applied nitrogen reaching a maximum at 45kg·ha-1 as indicated in Table 3 and Figure 3.1. Increasing nitrogenous fertilizer rates had negative effect on total phenolic content of baby spinach as seen on Table 3 and Figure 3.1 as total phenolic content were the lowest at 120 kg·ha-1N. This suggests that total phenolic content of baby spinach deteriorated with increasing nitrogenous fertilizer rates. The similar 78results were reported by Li et al., 2008 that total phenols decreased with increasing nitrogen application, the highest content was observed when the level of nitrogen was 100 kg·ha- 1 Clearly there was 26negative correlation between nitrogen application and total phenols content in the baby spinach leaves. The 26negative correlation between nitrogen application and Total phenolic content could be explained by the protein competition model (PCM) (Jones & Hartley, 1999). The PCM hypothesis makes a conditional prediction that there will be trade-offs between plant growth and Total phenolic content. This elucidates that 26when biomass increases in response to high nitrogen application, Total phenolic contents 26will decline because increased protein demand for growth will decrease dividing of carbon skeletons to Total phenolic contents (Jones and Hartley, 1999).4Haukioja et al., (1998) stated that the contradiction of the results of total phenolic content were largely due to lower leaf nitrogen content, seemingly due to increase in carbohydrate concentration when plants were stressed. According to Bryant et al., (1983, 1987) the carbon/nutrient balance (CNB) hypothesis 39suggest that growth is limited by deficiencies in carbon or nitrogen while rates of photosynthesis remain unaffected, and the following reduced growth results in the more abundant resource being invested in improved defence (mass-balance based allocation). Other studies showed when there is deficiency in N or N applications that significantly depressed yield resulted in the highest content of phenolic compounds in Chinese cabbage (Zhu et al., 2009), broccoli heads (Jones et al., 2007), lettuce (Coria- Cayupan et al., 2009). 4This suggests that there was a strong trade-off in nutrients directed toward the production of total phenols. 9The results showed that different nitrogen levels applied did not have any significant effect on the carotenoids content of baby spinach leaves (Table 3). This suggests that total carotenoid content did not respond to any level of N applied. These results are in contrary to the results reported in other studies, where the mid-range applications of N were most effective in lettuce at 0 – 1.6g·100g (Coria-Cayupan et al., 2009). Lefsrud et al., 2007 reported that 19lutein accumulations, expressed on a fresh weight basis, responded quadratically to increasing N treatments. The results showed that increasing nitrogen application increased the content of beta-carotene, but when the level of nitrogen was higher than 200 kg·ha-1, their contents remained nearly constant (Li et al., 2008). 13King et al., 2008 reported the effects of nitrogen and phosphorus fertilization on total carotenoid content in spinach and established that increases in nitrogen fertilization from 0 to 300 kg·ha-1 had slight effect on total carotenoid content. The effects of different nitrogen application rates on flavonoids are summarized in Table 3 and Figure 3.2, showing the range of applied N and the rate at which optimal flavonoid content was reached at 45kg·ha-1. In this study, it is notable that the lowest N application rate resulted in highest flavonoid content in baby spinach leaves. The results of the study suggested that the application of 45kg·ha-1 improved the total antioxidant activity content reaching 3.01 mg·g-1 (Table 3 & Figure 3.3). The total antioxidant activity content of 7plants under 45 kg·ha-1 was significantly higher compared to the control. It was further observed on the results that excessive Page | 66 application of nitrogen had a negative effect on total antioxidant activity in baby spinach. This was also reported by Mozafar, (1993) that higher levels of nitrogen increases concentration of nitrate (NO3) and simultaneously decreases the concentration of total antioxidant activity in vegetables. The similar results were reported by Briemer, (1982) and (Elia et al., 1999) that increased levels of nitrogen increased concentration of nitrate (NO3) while decreased the concentration of total antioxidant activity in vegetables. Kopsell 71et al., 2007 reported that nitrogen fertilization has effect on quality parameters leafy vegetables. Nitrate (NO3−) percentage in the baby spinach leaves increased with increasing nitrogen application reaching maximum of 1.40% at 120 kg·ha-1 (Table 3). The results of statistical analysis indicated that 57the effects of nitrogen levels on nitrate accumulation were significant (Table 3). It was observed an increase of the NO3− content, on the baby spinach leaves by using different nitrogen applications. This results are in agreement with Mozafar (1993) who reported that the over application 21of nitrogen fertilizers increases the concentration of NO3−. However, the nitrate contents in this study were acceptable as per World Health Organisation (WHO) standards. The WHO calculated the 29daily intakes of ≤500 mg of sodium nitrate per kg body weight were harmless to rats and dogs. This figure was divided by 100 to yield an Acceptable Daily Intake (ADI) for humans of 5 mg sodium nitrate or 3.7 mg nitrate per kg body weight, which equals 222 mg for a 60-kg adult. That figure has stood ever since Katan, (2009). Many 23vegetables and fruits contain 200–2500 mg of nitrate per kilogram (van Duijvenboden & Matthijsen, 1989). The nitrate content of vegetables can be affected by factors such as processing of the food, the use of fertilizers and growing conditions, especially the soil temperature and (day) light intensity (Gangolli et al., 1994; FAO/WHO, 1995). Vegetables such as beetroot, lettuce, radish and spinach often contain nitrate concentrations above 2500 mg/kg, especially when they are cultivated in greenhouses. (FAO/WHO, 1995). In addition, on another study reported that the concentration of nitrate-nitrogen in lettuce and spinach leaves is reliant on the rate of applied nitrogen fertilizer. For example, a significant increase of nitrates in lettuce and spinach was observed when N fertilizer application was increased from 260 to 280 kg·ha-1 (Szwonek, 1986). According to Khader, (2002) nitrogen fertilizers are known to increase the accumulation of nitrate concentration in leaves of most plants. The results of this study are in consistency with those reported by Hammad et al., (2007), Gulser, (2005), Elia et al., (1999) and Briemer, (1982) who also reported that the application of high nitrogen levels increases the accumulation of nitrate in vegetables. The results of this study clearly indicate that N applied had an effect on the Vitamin C concentrations in the baby spinach leaves. Vitamin C was quadratically increased by N application reaching maximum at 45 kg·ha-1 (Table 3 & Figure 3.4) but high rate of N application 21seem to decrease the concentrations of vitamin C in the baby spinach leaves. These results are in agreement with Mozafar, (1993), who reported that the increased levels of N application 21decrease the concentration of vitamin C in many fruits and vegetables. However, other reports by Kansal et al., (1981) and Dzida & Pitura (2008) showed that vitamin C and sugars content in spinach and Swiss chard increases under the application of high rates of nitrogen fertilizers. Lisiewska & 21Kmiecik (1996) stated that increasing the quantity of nitrogen nutrition from 80 to 120 kg·ha-1 decreased the vitamin C content by 7% in cauliflower. Likewise, Weston & Barth (1997) reported that N fertilization increases β-carotene in vegetables. 21Although Vitamin C concentration has been found to be positively associated with the nitrogen supply in butterhead lettuce (Muller & Hippe, 1987), it is inversely associated with the nitrogen21supply in white cabbage (Sorensen, 1984; Freyman et al., 1991) and crisp head lettuce (Sorensen et al., 1994). Although according to Uher et al., (2013), nitrogen fertilization positively influenced the bioactive compounds (vitamin C, E1 and ß-carotene) content in cauliflower edible heads. Increased fertilization doses of nitrogen significantly increased vitamin C and ß- carotene content in the cauliflower. This validates 7some recorded findings on fruits and vegetables by Skwarylo-Bednarz & Krzepilko (2008) who reported that the use of mineral fertilizer, mainly nitrogen increases the Vitamin C content. The 13plant scientist, Stamp, (2003) has suggested a model for explaining the relationship between nutrient availability in the soil and the growth and differentiation in plants called the growth-differentiation balance (GBD) hypothesis. This hypothesis proposes that the plant will allocate nutritive resources to growth if available in ample supply, but if resources are constrained the plant will divert more resources to the synthesis of secondary metabolites associated with differentiation. That means that if N is available, the plant diverts more input into N-containing metabolites, such as protein, vitamin A and nitrate, while more C containing compounds are produced when N is more restricted, such as vitamin C and many of the secondary metabolites. Stamp, (2003) demonstrated 13further that when resource availability is critically low both growth and secondary metabolite synthesis will be hampered. Nitrogen application significantly increase % leaf tissue N of baby spinach compared to control (0 kg·ha-1) reaching maximum at 75kgh·a-1. The highest percentage total leaf nitrogen was observed at 120 kg·ha-1, 3causing a linear response with high rates of nitrogen applied. (Table 3). 3Protein percentage seemed to have increased at 45kg ·ha which showed that there is a relationship between the nitrogen applied and protein found in the leaves of baby spinach. Increasing the rates of nitrogen applied to 120kg·ha did not exhibit protein content. 8Higher N fertilizer application levels increased the tissue N concentrations.26These results are in agreement with Hoque et al., (2010) who reported that a 8higher N fertilizer application increase the tissue N concentrations in lettuce. 24Wang et al. (2008) are that nitrogen fertilization increases the ratio of acids and sugars and reduces of vitamin C, calcium, magnesium and soluble sugars. Increased 24application of nitrogen reduced the content of dry matter, potassium, sucrose, vitamin C and fiber in leafy vegetables, but increased the content of nitrates and carotenes (Sorensen, 1999).

4.3.3 Response of chemical composition and trace elements to phosphorus nutrition in baby spinach

8Limited studies are available on the effect of P nutrients on baby spinach postharvest quality specifically chemical composition. What is known is that plants use phosphorus fertilizer for seed germination and root development and growth. Besides increasing the yield, potassium improves the quality of vegetables in several aspects, such as the chemical composition, the marketability and the storability of the crop King et al., 2008. In P trial, it was observed that different levels of phosphorus 9applied did not have any significant effect on the total carotenoid content in baby spinach leaves (Table 4). Thus, there was no significant increase on the concentration of carotenoids regardless of the levels of P applied.). On contrary to the findings of this study, 13King et al., 2008 reported the effects of nitrogen and phosphorus fertilization on total carotenoid content in spinach, and reported that the 13highest carotenoid contents were obtained with maximized phosphorous application reaching 50 kg·ha-1.74Based on these results it can be concluded that the increment of P fertilizer in baby spinach had a negative effect on the concentrations of bioactive compounds (total phenolic content, total flavonoid content, total antioxidant activity & Vitamin C). These results are consistent with those of earlier studies in which the secondary metabolite concentrations improved in P-deficient plants (John et al., 2009; Lei et al., 2011). It is clear that 33there is a lack of information on the effect of P fertilizer on the bioactive compounds on green leafy vegetables specifically baby spinach. In contrary other reports suggest that in crop such as lettuce, the results show a distinct 8yield and quality response to P fertilizer under most conditions (Alt, 1987; Johnstone et al., 2005; Sanchez and Burdine, 1988; Sanchez et al., 1988). Again, according to Soundy & Smith (1992) reported a significant positive linear correlation between soil P and head tissue P. This may be supported by the report of Cleaver & Greenwood (1975) that lettuce had 8higher P fertilizer requirements than most other vegetables across a range of soils. 4.3.4 Response of chemical composition and trace elements to potassium nutrition in baby spinach It has been reported that 51among plant nutrients K has the strongest impact on crop quality parameters that determine consumer preference (Jifon & Lester, 2009). K had been reported as an important macronutrient for plants and is needed for vital functions in metabolism, growth, and stress adaptation (Ismail et al., 1994; Rosa et al., 2001). Studies report different responses of vegetable crops to K nutrition. The 42known functions of K in solute carriage, protein synthesis, and enzyme activation indicate a close relationship between K and metabolism. As this study was conducted, it was established that 33there is a lack of information on the influence of K fertilizer on the concentrations of bioactive compounds in green leafy vegetable specifically baby spinach. Table 5 and figures 5.1 to 5.6 showed the variation in chemical concentrations in the leaves of baby spinach as affected by applied different levels of potassium nutrition. Based on 67the results of this study it is clear that K had influence on the bioactive compounds of baby spinach except on the trace elements and total carotenoid content. This concurs with the report that K is an important macronutrient for plants and is required for vital functions in metabolism, growth, and stress adaptation (Ismail et al., 1994; Rosa et al., 2001). Again, the results are in agreement with the report that 42K deficiency affects the metabolite concentrations in crops, with negative significances for nutritional quality and mechanical stability (Patrick et al., 2009). 4.3.5 Response of chemical composition and trace elements to nitrogen, phosphorus & potassium nutrition in baby spinach The applications of nitrogen, phosphorus and potassium have been a standard agronomic practice (Keen & Zidenberg-Cherr, 2000). 8Optimal fertilizer management and efficient use of N, P, and potassium (K) are necessary to improve yield and quality and to reduce production cost (Fageria, 2009). As this study was conducted, it was established that there is a lack of published reports 7on the influence of treatment combinations of NPK fertilizer on the concentrations of bioactive compounds in green leafy vegetable specifically baby spinach. There is limited information as far as this subject is concern. Some work has been done by other researcher's related crops such as lettuce and studies show 8that N availability in the soil can affect lettuce quality (Tittonell et al., 2001) but there is no evidence about the effect of different levels of N, P, and K on the postharvest quality of lettuce. Other studies reported that 8there may be some yield and quality benefit to N and P fertilizer application because of their role in moderating disease resistance and because of the metabolic functions of K (Marschner, 1995). This clearly indicate the shortage of information as far as NPK is concern in relation to bioactive compound of green leafy vegetables specifically baby spinach. From this study it can be concluded that regardless of different levels of NPK nutrition, there was no significant effect on trace elements (Mg, Fe, Zinc & Se) concentrations. However, the concentrations of other parameters such as total phenolic content, total antioxidant activity , total carotenoid content, total flavonoid content and Vitamin C in baby spinach seem to be influenced by the application of NPK. The optimum NPK level in this study was N45:P45:K60 kg·ha-1 where the total phenolic content, total carotenoid content, total antioxidant activity , total flavonoid content & vitamin C concentrations in baby spinach leaves were higher than all other levels. At N75:P75:K90 7kg·ha-1 the law of diminishing returns sets in. At this level all concentrations in bioactive compounds decreased due to different levels of NPK applied in the baby spinach leaves. At N45:P45:K60 7kg·ha-1 levels of NPK, total phenolic content and antioxidants concentrations of baby spinach leaves were highest (Tables 6). 7Further increase in the application of NPK resulted in over fertilization and subsequent effect of decline in the antioxidant activity and phenols and antioxidant activity constituents. This is in agreement with the study conducted in China by Juan et al., (2008) on effects of N and S on total phenolics and antioxidant activity of leaf Mustard. They reported that increasing N supply considerably decreased total phenolic concentrations. Similar findings were emphasized in addition to the type of soil in accumulation of nutrients by cultivated plants (Lester, 2007; Kadar, 2002; Kadar, 1988). The7effect of NPK on the anti-oxidant activities and components of baby spinach leaves is presented in (Table 6 & 6.4). The plants under N45, P45, K60 kg·ha-1 had the highest antioxidant activity of 6.5 mg·g-1 compared to other treatment combinations including the control (0 kg·ha-1). These results concur with 12Jeppsson, 2000; Kaur & Kapoor, 2001; Kemal et al., 2008 who reported that 12mineral fertilization influences antioxidant composition in some fruit and vegetables.4This resulted in the biosynthesis of carbon-based secondary metabolites, such as flavonoids, phenolic acids, and tannins, known as total polyphenols, which are antioxidant in nature (Haukioja et al., 1998). Nutrient-deficient plants often have lower growth rates and higher concentrations of carbon-based (non-nitrogen-containing) secondary compounds (CBSCs) than do plants with access to ample nutrients (Bryant et al., 1983; Coley et al., 1985). This negative correlation between concentrations of CBSCs and plant growth rate, or levels of nutrients in plant tissues, is assumed to indicate a trade-off between plant growth and the production of defensive compounds (Bryant et al., 1987). All treatment combinations of NPK rates applied on baby spinach did not show any significance effect on leaf tissue P percentage (Table 6). Increasing NPK fertilizer application rate had no significant effect on tissue P but had significant effect on N & K of baby spinach. 61These results are partially in agreement with the results reported by Hoque et al., (2010) that the increasing NPK 8did not have a significant effect on tissue P or K of lettuce. The treatment combinations of N30, P30, K45 kg·ha-1 improved the concentrations of leaf tissue N (%) (Table 6 & Figure 6.7). Percentage leaf tissue potassium was quadratically increased and ranged from 3.45 to 6.19% (Table 6 & Figure 6.9). The treatment combinations of N75, P75 & K90 kg·ha-1 improved the percentage leaf tissue K (Table 6 & Figure 6.9). The treatment combinations of N30, P30 and K48 (kg·ha-1) significantly improved leaf protein content percentage ranging from 20 to 27.8% (Table 6 & Figure 6.7). NPK had 19negative correlations between tissue%N & tissue%P.19Negative correlations between tissue%N and tissue%P have also been reported previously (Mills & Jones, 1996). CHAPTER 5 5.1 CONCLUSION AND RECCOMENDATIONS

Besides the maximization of yield in vegetable production research, the main goal these days is to produce healthy vegetables. Healthy vegetables referring to vegetables with optimal level of health promoting compounds such as total phenolic content, total carotenoid content, total flavonoid content, total antioxidant activity , vitamins etc. Minerals and bioactive compounds concentration in vegetables can be improved to some limits by best agricultural practices. Several factors influence the bioactive compound concentrations of baby spinach, including growing conditions, harvest time, individual plant characteristics, cultivation methods, cultivar, genetic factors and NPK fertilizers. It is clear that the best agricultural practices are the key in the improvement of health promoting compounds in vegetables and if all these factors are well considered, the concentrations on health promoting factors will increase. The study was conducted using Ohio cultivar, and this doesn't dismiss the fact that other cultivars might have responded differently to the NPK fertilizers. 3Based on the findings of this study, recommended rate of fertilizers in which baby spinach performs best regarding total phenolic content, total antioxidant activity, total flavonoid content and Vitamin C is 45kg/ha N and P. The recommended rate for K is 85 where total phenolic content, total antioxidant activity, total flavonoid content and Vitamin C concentrations are improved. Where treatment combination of 9NPK fertilizers was applied, the combination of 45:45:60kg/ha is recommended. Further studies to investigate the economic significance of these levels will be conducted.

There has been no response reported of magnesium (Mg), zinc (Zn), iron (Fe) & selenium (Se) on baby spinach to NPK nutrition, suggesting that this is a subject that still needs to be investigated. Fertilization can decrease or increase the level of concentrations on bioactive compounds depends on the applied level. Adequate levels are recommended in order for these compounds not to be affected negatively but positively in order to achieve the maximum concentrations in bioactive compounds of baby spinach. Finally it was concluded that fertilizer application had influence on bioactive compounds of baby spinach and adequate levels are critical in improving quality in baby spinach in terms of health related bioactive substances. If adequate levels are applied, bioactive compounds in baby spinach can be improved but excessive application of fertilizer has detrimental effect on the bioactive compounds of baby spinach based on the results of the study.

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APPENDIX B

ETHICAL CLEARENCE LETTER

UNISA university of south africa

2012-10-10

Ref. Nr.: 2012/CAES/033d

To the supervisor:
Prof FN Mudau
Department of Agriculture and Animal Health
College of Agriculture and Environmental Sciences

Dear Prof Mudau

Request for Ethical approval for the following research project:

Class Approval: Agronomic practices and nutritional profile and anit-cnacer molecules of baby spinach with reference to mineral nutrition

The application for ethical clearance in respect of the above mentioned research has been reviewed by the Research Ethics Review Committee of the College of Agriculture and Environmental Sciences, Unisa. Ethics clearance (Ref. Nr.: 2012/CAES/033d) **is granting Class Approval for the following research project entitled:** *Agronomic practices and nutritional profile and anit-cnacer molecules of baby spinach with reference to mineral nutrition*

Please be advised that the committee needs to be informed should any part of the research methodology as outlined in the Ethics application (Ref. Nr.: 2012/CAES/033d), change in any way. In this instance, a memo should be submitted to the Ethics Committee via Ms Marthie Van Wyk, in which the changes are identified and fully explained.

We trust that sampling, data gathering and processing of the relevant data will be undertaken in a manner that is respectful of the rights and integrity of all participants, as stipulated in the UNISA Research Ethics Policy.

The Ethics Committee wishes you all the best with this research undertaking.

Kind regards,

Prof E Kempen,
CAES Ethics Review Committee Chair

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