Growth, yield and chemical composition of *Pelargonium sidoides* DC. in response to nitrogen and soil water management

by

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Submitted in partial fulfilment of the requirements for the degree M.Inst.Agrar. (Agronomy)

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April 2015

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Declaration

I, Motiki Meshack Mofokeng, declare that the dissertation, which I hereby submit for the degree MIsntAgrar: Agronomy is my own original work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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ACKNOWLEDGEMENTS

For all the achievements in my life and studies "Glory be to the Almighty".

It gives me pleasure to acknowledge many individuals and organizations whom this study would not have been complete without their encouragement and contribution.

My sincere thanks and deepest gratitude goes to my supervisor Prof. J.M. Steyn and co-supervisors Dr C.P. du Plooy and Dr G. Prinsloo. Their support, patient guidance, encouragement and excellent advice throughout my studies are highly appreciated.

My grateful acknowledgment goes to the Department of Science and Technology, South Africa, for funding this study.

Many thanks go to all my colleagues, including N.R. Sathekge, K.A. Thaoge, T.S. Ramphisa, K.M. Nyathi, Dr Y. Beletse and Dr H.T. Araya for their support in different times of my study. Many thanks to A. Nkadimeng, S.O. Mailula, A.N. Maleka, C.R. Baloyi, G.K. Sethole and Z. Makhubela for their assistance in planting, maintaining and harvesting the trial. Mrs Itumeleng Setshedi (CSIR) is also thanked for her assistance with NMR analysis.

I am thankful to my wife Monicah, our son Toka and daughter Hali for all the love, encouragement and for allowing me to steal their time for my studies. This work is dedicated to my late parents Pheello Samson and Mamphakiseng Martha Mofokeng; and my late brother Tsoabetla Abram Mofokeng. I have not forgotten that no matter our background, you always believed that with education we can achieve what we aspire to. To my sisters Kgoabane, Mahadi and Mapaseka, let us "keep the candle burning forever".

"It does not matter where you are coming from, what matters is where you are going. So, work hard and shine on"

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GROWTH, YIELD AND CHEMICAL COMPOSITION OF *PELARGONIUM SIDOIDES* DC. IN RESPONSE TO NITROGEN AND SOIL WATER MANAGEMENT

M.M. Mofokeng

ABSTRACT

Pelargonium sidoides DC. is one of many medicinal plant species that are harvested in the wild. The current trade in these medicinal plants has negatively affected their distribution in the wild due to unsustainable harvesting. The demand for medicinal plants is unlikely to decrease, but sustainability of the supply is questionable. This is because commercial exploitation threatens to deplete their populations, resulting in many species being considered vulnerable to extinction and being lost from their natural habitat. Increased demand, which is already too high to be met by sustainable harvesting, and price increases presents potential opportunities for cultivating indigenous medicinal plants at a commercial scale. A need for basic information on how to grow the plants and other related matters necessitates that field trials be conducted, before farmers could be expected to venture into cultivating medicinal plants and this study aimed at developing such information.

The specific objectives of the study were to investigate 1) the physiological and morphological, 2) the yield components and 3) chemical composition of *P. sidoides* in response to soil water and nitrogen levels. The study was conducted under a rainshelter as a randomized complete block design with three irrigation levels, four nitrogen levels and three replicates. The irrigation treatments were 30% allowable depletion level (ADL) (well watered treatment), 50% ADL (moderately stressed treatment) and 70% ADL (severely stressed treatment), while the nitrogen levels were 0, 50, 100 and 150 kg · N · ha^{-1}. Dried root samples were analyzed for the presence of two standard compounds, scopoletin and esculin, using thin layer chromatography (TLC) and for metabolite profiling using the nuclear magnetic resonance technique (NMR).

Nitrogen and water level had no significant interaction effect on all measured parameters. Water stress significantly reduced stomatal conductance, while nitrogen had no significant effect on it. The well watered control had a significantly higher leaf area index, plant height and leaf area compared to the water stressed treatments. Nitrogen had a significant effect on the number of leaves, where 100 kg · N · ha^{-1} had a significantly higher number of leaves

compared to other nitrogen treatments. The well watered treatment again had a significantly higher total biomass, fresh and dry root yield; and nitrogen use efficiency compared to the water stressed treatments. The water use efficiency was significantly decreased in the well watered treatment. Water stress significantly increased nitrogen content and chlorophyll content of *P. sidoides* plants and leaves, respectively. Nitrogen levels of 50 and 100 kg · N · ha^{-1} resulted in a significantly higher total biomass compared to the control. TLC analysis showed the presence of the two standard compounds in all treatment samples analyzed. The orthogonal partial least square discriminatory analysis (OPLS-DA), which was performed on the NMR spectral data, showed separation between the irrigation treatments, resulting in two clusters representing the well watered treatment and the water stressed treatments. Asparagine, arginine, sucrose, xylose, glucose and citric acid were found to be the compounds associated with the separation. There was no separation of the samples regarding the nitrogen treatments which is indicative of the small effect of nitrogen on the metabolite content of the treatments. The results from this study showed a relationship between physiological, morphological and yield response as well as chemical composition of *P. sidoides*. The observed stomatal closure under water stress conditions, due to low turgor pressure in the guard cells, had a significant negative effect on leaf area, leaf area index, total biomass and root yield. Also the increases in total biomass and root yield under well watered conditions could be attributed to the increased primary metabolite content, under such conditions. The recommendation from the study is that *P. sidoides* plants should be grown under well watered conditions for a year or two to increase growth and root yield. Thereafter plants can be exposed to water stress in the second season, which is expected to increase the concentration of important secondary metabolites.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Background

Africa, and especially southern Africa, is rich in plant diversity with recent statistics showing that about 25% of the total number of higher plants in the world is found in Africa, south of the Sahara (Van Wyk, 2008). With over 19 500 indigenous plant species in about 350 plant families, South Africa has the richest temperate flora in the world (Crouch *et al*., 2008).

The herbal medicine industry was, in 2004, already worth more than US$ 60 billion and could be a natural answer to some human ailments (Magoro, 2008). It is for this reason that herbal medicines are growing in popularity in wealthy countries and their use remain widespread in developing countries (WHO, 2004).

1.2. African medicinal plant industry

Africa is well known as a rich source of medicinal plants and traditional rural African communities have relied upon the spiritual and practical skills of Traditional Health Practitioners for many years (Mativandlela, 2005). The traditional medicine trade in South Africa is a large and growing industry with about 27 million consumers and a contribution to the national economy was estimated at R2.9 billion already in 2007 (Sobiecki, 2014). Lewu *et al*. (2007) reported that 700 000 tonnes of plant materials were being harvested from the wild and consumed annually, with an estimated value of as much as 150 million US dollars per annum. Further reports are that at least 133 000 income earning opportunities were generated by the trade in traditional medicinal plants and their products in South Africa (Mander *et al*., 2007). There is a growing interest in natural and traditional medicines and scientists are looking for new cures in collaboration with traditional health practitioners (Mativandlela, 2005).

The current trade in medicinal plants in Africa has negatively affected the demography of most plant species harvested from the wild due to unsustainable harvesting (Lewu *et al*., 2006). The removal of whole plants, roots and bulbs totals 57% of all plant material traded in the Witwatersrand and may signify total loss of individual plants from the population (Dzerefos and Witkowski, 2001). Mander *et al.* (2007) reported that 86% of the plant parts harvested in the wild will result in the death of the entire plant and this has significant implications for the sustainability of supply. Ring barking and uprooting are the most common methods of collection used by commercial gatherers, which kills the plants. This is because the active ingredients are usually contained in the bark and roots of plants (Cunningham, 1993; Taylor *et al*., 1996). On the other hand, traditional harvesting methods that were more conservative and usually protected the plants to some extent, are now being ignored as the pressure to generate income exceeds concern for the resource base (Taylor *et al*., 1996).

O'Connor (2004) reported that the demand for medicinal plants is unlikely to decrease, but it is questionable whether continued harvesting can be sustained. This is because commercial exploitation threatens to deplete medicinal plant populations, resulting in many species being considered vulnerable to extinction and being lost from their natural habitat (Mativandlela, 2005). Finding a balance between natural resource use and conservation of these plants can become a challenge when dealing with the commercial use of wild resources (Motjotji, 2011). Canter *et al*. (2005) further indicated that approximately two thirds of the 50 000 different medicinal plant species in use are collected from the wild, with only 10% being cultivated. Species differ in their vulnerability to harvesting because of differences in demand, the manner of harvesting, the abundance of the species in the absence of harvesting and in their life history attributes (O' Connor, 2004). Vulnerability increases with low seedling survival, slow growth rates or long delays before harvestable size or reproductive maturity is reached (Dzerefos and Witkowski, 2001).

1.3. Rationale for the study

The importance of cultivation as a way of conserving medicinal plants cannot be ignored. Diminishes in supply and price increases presents potential opportunities for cultivating indigenous medicinal plants (Mander, 1998). The demand for medicinal plants is already too high to be met by sustainable harvesting and the only real solution would be to develop medicinal plants as crops through small-scale farming (Sparg *et al*., 2005). A need for basic information on how to grow the plants, planting to harvesting period and yield; and other related matters necessitates that field trials be conducted to establish this information, before farmers could be expected to venture into cultivating medicinal plants (Sparg *et al*., 2005).

The commercialisation of *P. sidoides* has led to indiscriminate harvesting of wild populations to meet the growing demand, particularly for the export market while another threat to wild populations is habitat loss due to the expansion of human settlements and the associated anthropogenic activities (Moyo *et al*., 2012).

Pelargonium sidoides has been harvested from the wild for decades by rural communities who depend on it for health purposes and also as a source of income. The current Government has introduced some interventions to help in dealing with the over-exploitation of the species, but this means cutting the main source of income for rural communities and in most cases leads to illegal harvesting and arrests. Commercial cultivation of *P. sidoides* will help in conserving the wild species while ensuring income for the rural communities. It is therefore very important to research the agronomic practices for *P. sidoides* which will increase yields while maintaining the medicinal value of the plant.

1.3.1. Study purpose and objectives

The increasing demand for *P. sidoides*, coupled with diminishing supplies from the wild, legal restrictions as well as the associated health and income benefits necessitate commercial cultivation of the species. It is therefore important that technical scientific information be developed on the cultivation of *P. sidoides*. Therefore the study aimed to develop such information.

It has been reported that soil water content, which has a direct influence on nutrient content, has an effect on chemical composition of *P. sidoides* and thus the focus of this study was on these two production factors.

The objectives of the study were as follows:

- To determine the effect of different nitrogen levels and soil water depletion regimes on growth, yield and chemical composition of *P. sidoides*
- To investigate the interaction effect of nitrogen and soil water depletion regimes on growth, yield and chemical composition of *P. sidoides*
- To start developing guidelines for nitrogen fertilization and irrigation management of *P. sidoides*

1.3.2. Hypotheses

i. There is an optimum N level (e.g. 100 kg · N · ha^{-1}) that will improve growth and increase yield of storage roots with no negative effect on medicinally active compounds of the plant.

ii. Improved root yield will lead to increases in total yield of the medicinally active compounds.

iii. Increasing nitrogen rates will significantly increase chlorophyll content and leaf area.

iv. Appropriate irrigation scheduling will improve yield of storage roots with no negative effect on active compounds, and thus increase the total yield of active compounds.

1.3.3. Structure of dissertation

This dissertation is divided into six chapters, as follows;

Chapter one (1) covers the general introduction and background of wild medicinal plants. The medicinal plant industry and cultivation of medicinal plants are discussed. The rationale for the study, purpose and objectives; and hypothesis are included in this chapter.

Chapter two (2) is the literature review, with specific details on the botany and distribution of *P. sidoides*, medicinal properties, harvesting and uses of *P. sidoides*; propagation and cultivation of the species.

Chapters 3, 4 and 5 present research results with each chapter focusing on specific research objectives. The chapters have been written in the format of papers for publication and having used this format, there are some repetition although efforts have been made to keep this to a minimum.

Chapter three is focused on the physiological and morphological response of *P. sidoides* to water and nitrogen level.

Chapter four presents the yield response of *P. sidoides* to water stress and nitrogen.

Chapter five provides an investigation into the chemical composition of *P. sidoides* in response to water stress and nitrogen level.

Chapter six is the general discussion on the findings of the study with concluding comments and final recommendations.

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CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

A few medicinal plants that are cultivated for the international trade industry include *Warburgia salutaris* (pepperbark tree), *Siphonochilus aethiopicus* (African ginger), *Agathosma* sp. (buchu), *Harpagophytum procumbens* DC. (devil's claw), *Pelargonium sidoides* DC. (rooi rabasam) and *Xysmalobium undulatum* (Uzara) amongst others (Mativandlela, 2005;Van Wyk, 2011). Many of these plants, though cultivated commercially, are also harvested in the wild, leading to over utilization of natural habitats (Mativandlela, 2005).

As supply of medicinal plants diminishes and prices increase, the potential opportunities for cultivating indigenous medicinal plants have increased substantially (Mander, 1998). With the increase in non-specialist gatherers and commercial traders, the demand for medicinal plants is already too large to be met by sustainable harvesting and the only real solution would be to develop medicinal plants as crops through cultivation (Sparg *et al.*, 2005). Sparg *et al*. (2005) mentioned that the need for basic information on how to grow the plants, planting time, harvesting period and yield, amongst others, necessitates that field trials be conducted to establish this information, before farmers could be expected to venture into cultivating medicinal plants. Cultivation of medicinal plants provides the potential to grow plants under environmental or physiological conditions that may promote the production of desired bioactive secondary metabolites (White, 2006).

Harvesting of medicinal plants for trade is a vital component of rural livelihoods, especially for women who are the most vulnerable sector of rural society and who may have no other means of income generation (Motjotji, 2011).

2.2. Pelargonium sidoides

Of the estimated 3000 medicinal plant species that are regularly used in traditional medicine in South Africa, only about 38 indigenous species have been commercialized to some extent (Van Wyk, 2008), while several others are wild harvested and in many cases over exploited for multi million Rand informal markets. Amongst these species which are unsustainably harvested and over exploited, is *Pelargonium sidoides* DC. which is in the Geraniaceae family (White, 2006; Lewu *et al.*, 2007a and Van Wyk, 2008). Brendler and Van Wyk (2008) reported *P. sidoides* as one of several geophytic species of the genus that are important traditional medicines in South Africa.

The Red List of South African plants (2009, 2011), lists *P. sidoides* as a ‘declining’ species in the conservation status and this means that although the plant is not yet critically endangered, there is a dire need for implementation of conservation measures (Moyo *et al.*, 2012).

2.2.1. Botany and distribution

P. sidoides is a small rosette-like perennial herb with tuberous rhizomes, sparsely branched stems, rounded to heart shaped leaves and small tubular flowers that are dark maroon red to almost black (van Wyk, 2008 and Lewu *et al.*, 2010). At full bloom, the species is about 20-50 cm high, forming a spiral of heart shaped leaves around its base (Lewu *et al.*, 2010).

Morphologically it is very similar to its close relative *Pelargonium reniforme*, but can be readily distinguished by its dark red rather than pink petals (Lewu *et al.*, 2007b). The sepals of *P. sidoides* are green with white margins, while those of *P. reniforme* are red with pink margins (White, 2006). The leaves of *P. sidoides* are velvety, cordate, heart-shaped and long-stalked, whereas those of *P. reniforme* are kidney shaped (Bladt and Wagner, 2007; Adewusi and Afolayan, 2009) and have crenate or finely lobed margins (Kolodziej, 2002). *P. sidoides* have yellow-green pollen whereas *P. reniforme* has whitish-green pollen (Bladt and Wagner, 2007). Although the shape of leaves and color of flowers can distinguish between the two species (Figure 2.1. A, B, D and E), the existence of gradual variations needs to be acknowledged (Kolodziej, 2002 and Lewu *et al.*, 2007b).

Figure 2.1. Dark maroon flowers (A) of *P. sidoides* as compared to the pink flowers of *P. reniforme* (D). The leaves of *P. sidoides* (B) and those of *P. reniforme* (E). Tuberous roots of *P. sidoides* (C) with their internal reddish colour (F).

P. sidoides usually grows in short grassland and sometimes on stony soil, varying from sand to clay-loam, shale or basalt; and it is usually restricted to the Grassland and Savanna biomes and Grassy fynbos (Mativandlela, 2005 and Motjotji, 2011). The thickened underground root system (Figure 2.1. C) penetrates deep into the ground and seem to be a special adaptation which enables the plant to survive grass fires which occurs throughout much of its habitat range (Motjotji, 2011). The roots of *P. sidoides* start off pale pink and as the plant matures turn deep red (Figure 2.1. F) and it is only at this stage that it has any commercial value (Stern, 2008). It grows naturally in summer rainfall areas with average annual rainfall ranging from 200 to 800 mm per annum (Mativandlela, 2005).

The species occurs naturally in Lesotho as well as the Eastern Cape, Free State, Northwest, Gauteng and Mpumalanga Provinces of South Africa (Figure 2.2) (Brendler and van Wyk, 2008), thus it can tolerate a wide range of environmental conditions (Motjotji, 2011). *P. reniforme* has a much narrower distribution range and is more or less confined to the Eastern

Cape Province (Brendler and van Wyk, 2008). *P. sidoides* is used locally in the Eastern Cape for the treatment of several cold related ailments, in man and livestock. There has been a significant increase in demand for the plant for both local uses and international pharmaceutical producers in recent years, resulting in an increase in the number of collectors and rate of wild harvesting (Lewu *et al.*, 2007a). The commercial demand for *P. sidoides* tubers for local and international trade has caused an enormous increase in the rate of uncontrolled, illegal and indiscriminate wild harvesting, which is fast leading to irreparable reductions in the number of natural populations (Colling *et al.*, 2010). Two popular articles in newspapers and one online publication reported uncontrolled harvesting of at least 20 tonnes of *P. reniforme* and *P. sidoides* roots in the Eastern Cape in 2002 (White *et al.*, 2008).

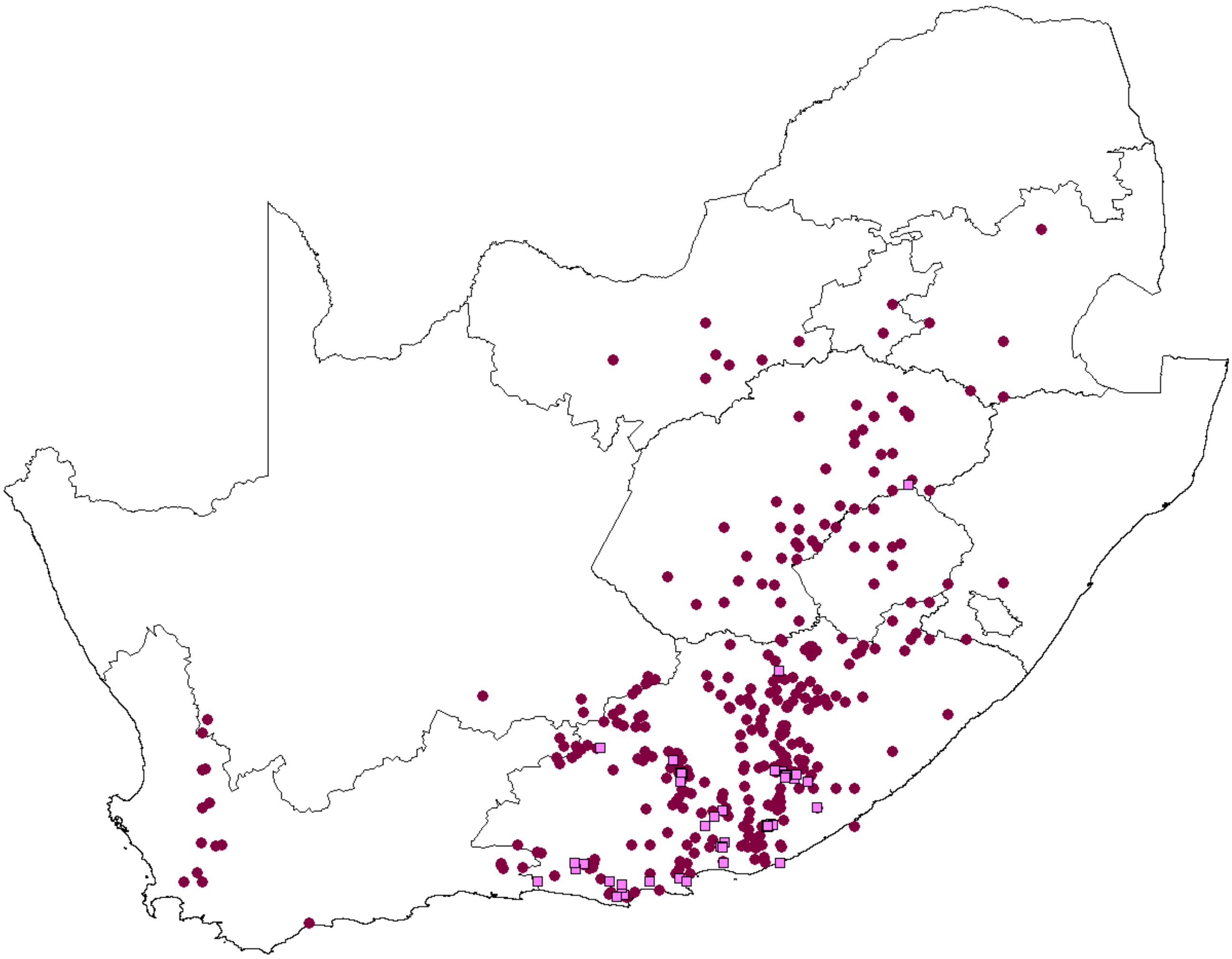

Figure 2.2. Geographical distribution of *P. sidoides* (maroon circles) and *P. reniforme* (pink squares) in South Africa. Source: SANBI distribution data (2013).

2.2.2. Bio-activity and uses of *P. sidoides*

Various studies have been done on the activity of *P. sidoides* extracts against acute bronchitis and common colds (Golovatiouk and Chuchalin, 2002; Chuchalin *et al.*, 2005; Lizogub *et al.*,2007; Agbabiaka *et al.*, 2008 and Bachert *et al.*, 2009); on the antibacterial, antifungal and antitubercular activity (Kolodziej *et al*., 2003; Seidel and Taylor, 2004; Mativandlela, 2005; Lewu *et al*, 2006; Conrad *et al*., 2007; Mativandlela *et al.*, 2007; Wittschier *et al.*, 2007 and Kim *et al.*, 2009); antimicrobial, antiviral and immunomodulatory activity (Kayser *et al*., 2001; Kolodziej and Kiderlen, 2007; Schnitzler *et al.*, 2008 and Kolodziej, 2011). Bladt and Wagner (2007), gives a good account of the history of *P. sidoides* in curing tuberculosis.

The fleshy bright red tubers have been widely used to treat diarrhoea and dysentery (Kolodziej and Kiderlen, 2007; Lewu *et al.*, 2007a; Brendler and van Wyk, 2008); bronchitis (Chuchalin *et al.,* 2005 and Van Wyk, 2011), cough, fever, tuberculosis, sore throat, fatigue and weakness of the body (Mativandlela, 2005; Lewu *et al.*, 2006; White *et al.*, 2008; Van Niekerk and Wynberg, 2012).

The tuberous roots are the raw material for an important German phytomedicine (Lewu *et al,* 2007a and Van Wyk, 2008) and many other herbal medicine products, which are approved and registered drugs in countries such as Turkey, Mexico, Brazil, Austria, Belgium, Italy and many others, for the treatment of common colds, acute bronchitis and infections of the upper respiratory tract (Chuchalin *et al.*, 2005; Kolodziej, 2007; European medicines agency, 2012; Street and Prinsloo, 2013). In Cape Town, South Africa, a cough remedy called Linctagon (Figure 2.3) is manufactured for the local market (Stern, 2008). There is a possibility that the key ingredient may be revived as a cure for TB, particularly after the rise of drug-resistant strains of the disease (Stern, 2008). The annual sales of *P. sidoides*, one of the ten commercially important medicinal plants of South Africa, exceed € 80 million in Germany alone and most of the material used is still harvested from the wild by locals from rural communities in South Africa (Street and Prinsloo, 2013).

Figure 2.3. Some of the pharmaceutical products, made from root extracts of *P. sidoides* that are available over the counter in South Africa. Source: www.nativa.co.za

2.2.3. Wild harvest of *P. sidoides*

Most of the gatherers in the Eastern Cape are from rural communities, with no other source of income and thus they rely heavily on natural resources for sustenance (Lewu *et al.*, 2007a; Van Niekerk and Wynberg, 2012). In their findings, Lewu *et al.* (2007a) reported that more than 26 tonnes of *P. sidoides* were collected and an estimated $14 000 worth of the species sold from 10 settlements in the Eastern Cape, in just one month. Although harvesting and sales of *P. sidoides* from the wild brings financial returns to the people, the consequences of uncontrolled harvesting may affect its availability for future generations (Lewu *et al.*, 2007a). All the respondents in the mentioned study (Lewu *et al.*, 2007a) agreed that *P. sidoides* was becoming more difficult to find in the wild. The Eastern Cape Department of Economic Development and Environmental Affairs, in June 2006, responded to over-exploitation of pelargonium by placing a temporary ban on harvesting and exporting of *P. sidoides* and *P. reniforme* from the wild, and similar safeguards have also been placed by the Lesotho Government (African Centre for Biosafety, 2008). The pressure to supply the roots has prompted illegal harvesting, resulting in several illegal harvesters being arrested in the Eastern Cape and in Lesotho, already in 2008 (African Centre for Biosafety, 2008).

Lewu *et al.* (2007a) observed that the rate of harvesting does not support annual collection, as the second year of a regeneration study gave less than 50% biomass yield compared to the initial harvested mass and therefore recommended that the species be left undisturbed for some years after harvesting to give the population time to recover. Motjotji (2011) also reported that

P. sidoides roots may require 10 – 15 years of recovery to reach the same pre-harvest tuber mass, thus any harvesting before the suggested period has elapsed could have a negative effect on wild populations.

Harvesters collect large tubers with dark-red colouration as these tubers are believed to be more potent and thought to have higher concentrations of the desired medicinal compounds (Motjotji, 2011). However, White (2006) found no direct relationship between darkness of colour and umckalin concentration in dried roots of *P. sidoides*. Motjotji (2011) argues that the lack of any significant relationship between root colour and umckalin concentration may be due to the use of a single extract (umckalin) by White (2006), as opposed to a complex of extracts reported by the Pelargonium industry. It is suspected that umckalin may not be an active compound of *P. sidoides* but just a marker compound to differentiate between roots of *P. sidoides* and those of *P. reniforme*. Furthermore, although *P. reniforme* does not have umckalin, both the roots of *P. sidoides* and *P. reniforme* are traditionally used for the same purposes (Kolodziej, 2002; White, 2006 and White *et al.*, 2008). Mativandlela (2005) found that the roots of *P. reniforme* showed inhibitory activity against *M. tuberculosis*. The same study justified the traditional use of the two *Pelargonium* species in the treatment of lung and respiratory tract infections. *P. sidoides* has significantly higher yield of coumarins than *P. reniforme.* The total coumarin content of *P. sidoides* roots is approximately 0.05% related to dry weight, with umckalin accounting for about 40% of total coumarin content (European medicines agency, 2012), which could be a possible reason why *P. sidoides* roots are preferred more than those of *P. reniforme*. The coumarin pattern of EPs®, a proprietary root extract contained in one of the herbal products Umckaloabo®, was strongly reminiscent of that of *P. sidoides* (Gödecke *et al*., 2005 and Kolodziej, 2007).

2.2.4. Propagation and cultivation

Although *P. sidoides* tubers are highly sought-after, they are only cultivated on a small, virtually negligible scale (Colling *et al*., 2010). Some of the reasons for the small scale plantation include economies of scale and slow regeneration rate of the plant (Van Niekerk and Wynberg, 2012). To secure a regular supply of *P. sidoides* large scale cultivations were initiated by Schwabe, a German Pharmaceutical company, in Kenya and Mexico, when permitting arrangements were becoming stricter in southern Africa (Van Niekerk and

Wynberg, 2012). Van Wyk (2011) mentions suitability for cultivation as one of the important aspects when selecting species with commercial potential as herbal products.

Lewu *et al.* (2010) found that the germination of *P. sidoides* seeds is dependent on light, temperature, pre-chilling, seed age and source of seed; and this could be a possible explanation for the low rate of regeneration by seed in the wild (Lewu *et al.*, 2006). They suggested that aerial vegetative parts, which are normally discarded during harvesting, can be used for propagation of this species (Lewu *et al.*, 2006).

Naturally, regeneration of the species is by seed and from development of perennates through an underground root system (Lewu *et al.*, 2006). However, viability of the seeds collected from the wild is low, coupled with very low seed germination and during harvest it is the roots that are removed, threatening the opportunity for perennial rejuvenation (Lewu *et al.*, 2006). Canter *et al.* (2005) also mentioned that cultivation of some herbal plants has proved to be difficult because of low germination rates, specific ecological requirements or lack of knowledge. The increased rate of harvesting and number of plant gatherers has made its exploitation unsustainable (Lewu *et al.*, 2006).

Lewu *et al.* (2006) reported that cultivation of *P. sidoides* could be an alternative source of supply in order to take pressure off the wild stock. The same sentiment is shared by White *et al.* (2008), mentioning that commercial cultivation of pelargonium has distinct advantages which would be ensuring a supply of roots of the correct species that have documented medicinal efficacy, and that the use of irrigation can increase the biomass yield and decrease chemical variability of plants. One of the practical measures against over-exploitation of plant natural resources is to improve propagation and cultivation (Lewu *et al.*, 2006). In the best interest of conserving *P. sidoides* and the sustainable supply of roots for local and international markets, White *et al.* (2008) strongly recommended that cultivation be further researched and pursued as a promising alternative to wild harvest.

However, the challenge with propagation and cultivation of *P. sidoides* is that plants propagated from the wild stock revealed that the concentration of umckalin in their roots was significantly lower than that of wild plants, while the concentration of cultivated non-clonal plants compared to that of wild plants (White, 2006). In analyzing root extracts from different regions, White (2006) revealed that average root umckalin concentrations were strongly related to both the average soil pH and annual rainfall of the collection sites. The study further found that prolonged water stress treatments did not significantly alter the root umckalin concentrations of cultivated plants relative to well-watered controls. Furthermore, the umckalin concentration in new roots of cultivated plants was not significantly different to that of old roots collected in the wild (White, 2006).

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CHAPTER 3

PHYSIO-MORPHOLOGICAL RESPONSE OF *PELARGONIUM SIDOIDES* DC. TO SOIL WATER AND NITROGEN LEVEL

Abstract

Water stress is the most limiting factor in agricultural productivity in arid and semi-arid regions and causes very high losses in crop yield. Regulation of growth and stomatal conductance are the main mechanisms by which plants respond to water stress. *Pelargonium sidoides* is a medicinal plant that grows in South Africa and is used for the treatment of upper respiratory ailments. Cultivation has been considered as a viable means of reducing the pressure on natural populations of this species, but little to or no information is available in this regard. Water and nitrogen supply are two of the most important factors that affect growth and yield of plants. This study therefore aimed at investigating the physiological and morphological response, in relation to growth, of *P. sidoides* to soil water and nitrogen levels. To achieve this objective *P. sidoides* plants were grown under a rainshelter and exposed to three irrigation levels (well watered control, moderate water stress and severe water stress treatment) and four nitrogen levels (0, 50, 100 and 150 kg · N · ha^{-1}). Nitrogen and water level had no significant interaction effect on all measured parameters. Water stress significantly reduced stomatal conductance, while nitrogen had no significant effect on it. The well watered control had a significantly higher leaf area index, plant height and leaf area compared to the water stressed treatments. Nitrogen had a significant effect on the number of leaves, where 100 kg · N · ha^{-1} had a significantly higher number of leaves compared to other nitrogen treatments. The study provides a first report on the response of *P. sidoides* to water and nitrogen; and showed that the plant responds to water stress by closing its stomata and employing other morphological strategies like reducing plant growth. These results will assist in explaining yields under different irrigation and nitrogen management strategies in cultivation of *P. sidoides*.

Keywords: allowable depletion, growth response, nitrogen, *Pelargonium sidoides*, stomata, water stress

This chapter has been published in the South African Journal of Botany:
M.M. Mofokeng, J.M. Steyn, C.P. du Plooy, G. Prinsloo, H.T. Araya. 2015. Growth of *Pelargonium sidoides* DC. in response to water and nitrogen level. *S. Afr. J. Bot.* 100: 183-189

3.1. Introduction

Pelargonium sidoides, which grows naturally in South Africa, is used in the Eastern Cape Province for the treatment of several cold related ailments in humans and livestock (Lewu *et al.*, 2006; Brendler and van Wyk, 2008). Furthermore, Helfer *et al*. (2014) also proposed that *P. sidoides* root extract has shown anti-HIV-1 properties. Up to now *P. sidoides* plant material for medicinal use has almost exclusively been harvested from the wild. However, there has been an increase in demand for the plant for traditional use as well as by local and international pharmaceutical companies (Lewu *et al.*, 2007). As a result, cultivation has been considered as a viable means of reducing the pressure on natural *P. sidoides* populations. Information on the cultivation of medicinal plants such as *P. sidoides* is, however, very limited and therefore further research was needed.

Water and nutrient supply are two of the most important factors that can affect growth, biomass yield and chemical composition of plants and therefore this study focused on these two production factors. Exchange of water and carbon dioxide (CO_2) between leaves and the ambient air are important plant processes by which heat is dissipated through transpiration, while a primary substrate for photosynthesis is taken up (Streck, 2003). The ability of plants to adjust gaseous exchange through stomata permits them to control water relations and carbon assimilation; and the opening of the stomatal pore reflects a compromise between the photosynthetic requirement for CO_2 and the availability of water (Tricker *et al*., 2005). Regulation of leaf expansion and stomatal conductance are the main mechanisms by which plants respond to soil water deficit (Liu and Stützel, 2002; Eiasu *et al*., 2012). High transpiration causes stomatal closure, possibly by increasing the water potential gradient between the guard cells and other epidermal cells or by lowering the leaf water potential, either of which directly decrease the turgor pressure of guard cells relative to other epidermal cells or affect hormonal distribution (Bunce, 1996).

Water deficit in plants leads to physiological disorders, such as a reduction in photosynthesis and transpiration (Petropoulos *et al*., 2008); however the effects vary between species (Karkanis *et al*. 2011). Both photosynthesis and transpiration, which are closely related to dry matter production, are regulated by a stomatal feedback control mechanism which, in turn, is influenced by water deficits (Kumar *et al*., 1994 and Bota *et al*., 2004). The limitation of plant growth enforced by low water availability is mainly due to decreases in plant carbon balance, which is dependent on the balance between photosynthesis and respiration (Flexas *et al*., 2006). An early response to water stress supports immediate survival, whereas acclimation, calling on new metabolic and structural capabilities mediated by altered gene expression, help to improve plant functioning under stress (Chaves *et al*., 2002). Shoot growth is more sensitive to water deficit than root growth and the mechanisms underlying the sustained root growth under water stress include osmotic adjustment and an increase in the loosening capacity of the cell wall (Chaves *et al*., 2002).

Deficit irrigation is becoming an important strategy to reduce agricultural water use in arid and semi-arid regions (Ayana, 2011). It is the practice of deliberately under irrigating crops to reduce water consumption while minimizing adverse effects of extreme water stress on yield (Ayana, 2011). Deficit irrigation does not always decrease yield, as deficits properly applied in some development stages may even increase crop yield (Bilibio *et al*., 2011).

Nitrogen increases leaf area index (LAI) and also improves the physiological properties of the plant (Kara and Mujdeci, 2010). Nitrogen is a component of many biological compounds that plays a major role in photosynthetic activity and crop yield capacity; and its deficiency constitutes one of the major yield limiting factors for production (Hokmalipour and Darbandi, 2011). Nitrogen deficiency leads to loss of green colour in the leaves, decreased leaf area and intensity of photosynthesis, leading to reduced photosynthate production and thus lower yields (Alva, *et al*., 2006, Bojović and Marković, 2009). Leaf area influences the interception and utilization of solar radiation of crop canopies (Hokmalipour and Darbandi, 2011), but it also plays an important role in water use (Liu and Stützel, 2002). Leaf area index is a significant feature for the determination of plant photosynthetic activity and is a crucial structural characteristic of plants due to the role of green leaves in controlling many biological and physical processes in plant canopies (Kara and Mujdeci, 2010). Over application of nitrogen causes many environmental pollution problems (Lee *et al*., 2011) and can lead to decreased yields due to luxury consumption (Alva *et al*., 2006).

Plants take up inorganic nitrogen contained in the water absorbed from soil solution through their root systems and thus, the fate of nitrogen is certainly coupled to that of water reaching the soil in the root zone (Alva *et al.*, 2006). Water and nitrogen deficiency induces alterations of many morphological and physiological processes (Shangguan *et al*., 2000). Information on the response of *P. sidoides* to different water stress and nitrogen deficiency levels is not known and thus the objective of this study was to investigate the effect of water stress and nitrogen level on the physiology and morphology of *P. sidoides*.

3.2. Materials and methods

3.2.1. Study area

A field trial was conducted under a rainshelter at the Agricultural Research Council-Roodeplaat Vegetable and Ornamental Plant Institute (ARC-Roodeplaat VOPI), Pretoria, South Africa (25° 59'S ; 28° 35'E and 1 200 m.a.s.l.) during 2012 and 2013. The rainshelter is designed to automatically open when there is no rain and close during a rainfall event, thus excluding rainfall from the experiment. The physical and chemical properties of the soil at the experimental site are shown in Tables 4.1 and 4.2, respectively, while a summary of the weather data recorded by a weather station (Campbell Scientific, USA) at the experimental site during the experiment period is shown in Table 3.3.

Table 3.1. Physical properties of the soil at the experimental site, per 20 cm depth increment to a depth of 100 cm.

Soil depth (cm)	Sand	Silt	Clay	Texture	PWP	FC	BD (mg/m^3)
	%						
0 - 20	78	6	16	sandy loam	10.3	19.9	1.59
20 - 40	70	8	22	sandy clay loam	12.9	25.5	1.56
40 - 60	64	8	28	sandy clay loam	15.2	25.3	1.45
60 - 80	62	8	30	sandy clay loam	14.7	28.0	1.38
80 - 100	60	8	32	sandy clay loam	15.1	28.1	1.35

*PWP – permanent wilting point, FC – field capacity, BD – bulk density

Table 3.2. Chemical properties of the soil at the experimental site.

Soil depth (cm)	Fe	Mn	Cu	Zn	Ca	Mg	Na	P	K	Total N	pH H_2O
	mg/kg									%	
Top soil (0–20)	13.74	44.10	9.24	14.00	980	298	24.7	80.9	134	0.028	7.26
Sub soil (20–40)	9.74	28.50	5.64	7.43	1201	370	39.4	60.4	94	0.026	7.44

Table 3.3. Summary of weather data collected during the experiment period.

Month (2013)	Temperature (°C)		Wind speed (ms^{-1})	Relative humidity (%)		VPD (kPa)	Rainfall	ET_0
							mm	
	Average						Total	
	max	min		max	min			
Jan	30.73	16.85	0.90	87.45	34.61	1.28	90.3	168.0
Feb	32.06	15.77	0.81	88.80	27.89	1.40	35.0	159.2
Mar	29.53	14.43	0.75	89.78	30.54	1.11	75.9	133.2
Apr	26.10	9.21	0.67	91.70	32.39	0.89	98.2	107.6
May	24.83	4.97	0.59	89.73	23.43	0.87	0.55	95.3

*Max: maximum, min: minimum, VPD: vapor pressure deficit.

3.2.2. Trial design, plant material and duration

The trial was a randomized complete block design with three replicates and 12 treatment plots per replicate. The treatment factors were water and nitrogen levels. Each treatment plot was 4.5 m^2 in size, with 30 plants planted at a spacing of 0.5 m between the rows and 0.3 m in the row, giving a total plant population of 66 666 plants · ha^{-1}.

The mother material was acquired from a nursery at the Golden Gate Highlands National Park, in the Free State province of South Africa, in 2010 and grown under shade-net (grey, 40% shade effect) at ARC-Roodeplaat VOPI. Root cuttings were made from the mother plants between January and February 2012. Four months old rooted cuttings of *P. sidoides* were transplanted into the rainshelter in May 2012 and were established for six months, to ensure a good stand, before application of the treatments. The trial was harvested in June 2013.

3.2.3. Treatment application

Irrigation treatments were scheduled based on the allowable depletion levels (ADL) of the plant available water (PAW) in the soil. From previous observation the effective rooting depth (ERD) was determined as 400 mm. The predetermined treatments applied were 30% (well watered treatment), 50% (moderately stressed treatment) and 70% ADL (severely stressed treatment) of PAW, where a specific percentage of PAW was allowed to deplete from the ERD before filling the soil profile back to field capacity. A neutron probe (Waterman, Probe Version 1.6, 2005, Geotech) was used to monitor soil water content twice a week. Neutron probe readings were taken at intervals of 0.2 m to a soil depth of 1.0 m, through aluminum access tubes that were pre-installed in each plot. The instrument was calibrated to a depth of 1.0 m, at intervals of 0.2 m and calibration functions were developed following the method described by Evett (2008). These calibration functions are given in Appendix 2.

The allowable soil water deficits for the three water levels were calculated as follows and they were based on the field capacity (FC), permanent wilting point (PWP) and bulk density (BD) as shown on Table 3.1.

$$\begin{aligned}
PAW_{(0-200\text{ mm})} &= ((FC - PWP) \text{ x } BD) \text{ x } 200 \text{ mm} \\
&= ((0.199 - 0.103) \text{ x } 1.59) \text{ x } 200 \text{ mm} \\
&= \underline{30.5 \text{ mm}}
\end{aligned}$$

$$\begin{aligned}
PAW_{(200-400\text{ mm})} &= ((FC - PWP) \text{ x } BD) \text{ x } 200 \text{ mm} \\
&= ((0.255 - 0.129) \text{ x } 1.56) \text{ x } 200 \text{ mm} \\
&= \underline{39.3 \text{ mm}}
\end{aligned}$$

$$\begin{aligned}
\text{Total PAW for ERD}_{(400\text{ mm})} &= 30.5 \text{ mm} + 39.3 \text{ mm} \\
&= \underline{69.8 \text{ mm}}
\end{aligned}$$

$$\begin{aligned}
30\% \text{ ADL of PAW} &= 69.8 \text{ mm x } 30\% \\
&= \underline{20.94 \text{ mm}}
\end{aligned}$$

$$\begin{aligned}
50\% \text{ ADL of PAW} &= 69.8 \text{ mm x } 50\% \\
&= \underline{34.9 \text{ mm}}
\end{aligned}$$

$$\begin{aligned}
70\% \text{ ADL of PAW} &= 69.8 \text{ mm x } 70\% \\
&= \underline{48.86 \text{ mm}}
\end{aligned}$$

A computerized, non-pressure regulated, drip irrigation system (NETAFIM, South Africa) with a discharge rate of 2 L per hour and maximum pressure of 270 kPa was used for irrigation. Soil samples were taken in the field to determine the soil properties and the nutrient status of the soil which are given in Tables 4.1 and 4.2 respectively.

The nitrogen (N) treatments were different levels of N, applied at the following rates; control (0), 50, 100 and 150 kg · N · ha^{-1}. The N source used was Limestone ammonium nitrate (LAN 28% N), applied in two split applications of 50% each. The first N application was four months after planting, followed by the second application eight weeks after the first application.

3.2.4. Agronomic practices

As a base application of potassium (K) and phosphorus (P) were applied and worked into the soil five days after planting, while nitrogen (N) was applied six weeks after planting, based on soil nutrient status and estimated nutrient requirements of rose-scented geranium (*Pelargonium capitatum*) (Araya *et al*., 2006), since there was no recommendation available for *P. sidoides*. Potassium (K) was applied as potassium chloride (50% K) at a rate of 110 kg · K · ha^{-1} and P was applied as single-super phosphate (11% P) at a rate of 30 kg · P · ha-^{1}. Before nitrogen treatments were initiated, two weeks after planting, all plots were fertigated with potassium nitrate (13 % N, 38 % K) at a rate of 150 kg · N · ha^{-1} as a once off to boost plant establishment.

Weed control was performed manually with hand hoes. Servus (active ingredient: deltamethrin (pyrethroid)) was applied for pest control only when pests were identified after scouting.

3.2.5. Data collection and statistical analysis

Leaf area index (LAI) was measured non-destructively, using a LAI 2200 plant canopy analyzer (Li-Cor Bioscience, USA). The instrument uses measurements made above and below the canopy to calculate light interception at five zenith angles, from which LAI is computed using a model of radiative transfer in vegetative canopies. One above canopy reading and four below canopy readings were taken using the 270° view cap; and this was replicated two times in each plot. Plant height (cm) was measured and the number of leaves were counted manually. LAI and plant height measurements were taken on a monthly basis after treatment implementation. After harvesting, the total leaf area per plant was measured with a leaf area meter (Li-3100, Li-Cor Inc., Lincoln, USA).

Stomatal conductance, which is a measure of the rate of passage of carbon dioxide (CO_2) or water vapour through the stomata of a leaf, was measured using the SC-1 Leaf porometer (Decagon Devices, USA), on a monthly basis. Stomatal conductance is described as a function of the density, size, and degree of opening of stomata. The measurements were taken on the abaxial (bottom) side of a fully expanded mature leaf, during midday when the environmental factors were at their peak.

Data was subjected to analysis of variance (ANOVA) using *GenStat*® version 11.1 (Payne *et al*., 2008). Treatment means were separated using Fisher's protected T-test for least significant differences (LSD) at 5% level of significance (Snedecor and Cochran, 1980).

3.2.6. Scanning Electron Microscope (SEM) observations

Three leaf samples were collected and observed under an electron microscope to observe the stomata, following the method described by Motsa (2006) and Eiasu *et al*. (2012). The samples comprised of young, mature and old leaves. Samples (10 mm x 10 mm) were cut from each leaf sample and fixed in glutaraldehyde (3% w/v) immediately after cutting it from the plant. They were then rinsed thoroughly with a phosphate buffer (0.1 M, pH 7.0) for 15 minutes, and repeated three times. Thereafter the samples were dehydrated in ethanol series (30 – 100% w/v) and then dried in a critical point drying apparatus (Bio-Rad E300, Watford, England). The dried samples were mounted on copper stubs and coated with gold in a vacuum coating unit (Polaron E5200C, Watford, England). The samples were then observed under a JSM 840 scanning electron microscope (JEOL, Tokyo, Japan) at 2000X magnification.

3.3. Results and discussion

3.3.1. Growth response

Nitrogen and water level had no significant interaction effect on the growth parameters measured. Table 4 shows the average LAI, plant height and number of leaves per plant (means across treatments) taken from seven months after planting until 11 months after planting, which was four months after water treatment application. The average LAI, plant height and number of leaves per plant decreased over the growing period across all water treatments (Table 4). The results in Figure 1, further shows that the LAI for the well watered control (30% ADL) dropped slightly after one month of water treatment application, but thereafter there were no further significant reductions. The well watered control always had a significantly higher LAI throughout the growing period (Figure 3.1). Though the severely stressed treatment (70% ADL) had the lowest LAI throughout, it was not significantly different from the moderately stressed treatment (50% ADL). A number of studies on different crops have also reported a decline in LAI values due to water stress. In their studies, Eiasu *et al*. (2008, 2009) also found that the LAI of rose-scented geranium was negatively affected by water stress, with a significant decline in LAI between the well watered control and the water stressed treatments.

Laurie *et al*. (2009) found a large reduction in LAI, of about 64 - 80%, due to reduced irrigation on different sweet potato varieties.

Table 3.4. Averages of plant height, number of leaves and leaf area index of *P. sidoides* over the growth period.

Growth period (MAP*)	LAI (m^2 leaf area/m^2 ground area)	Plant height (cm)	Number of leaves/plant
7	1.40[a]	-	-
8	1.18[b]	17.40[a]	173[a]
9	1.08[bc]	17.80[a]	162[b]
10	0.95[cd]	16.50[b]	153[c]
11	0.88[d]	14.30[c]	123[d]
LSD$_{0.05}$	0.13	0.65	9.07

*MAP = month after planting. Values with different letters are significantly different from each other. Values represent means across all water treatments. LSD: least significant differences.

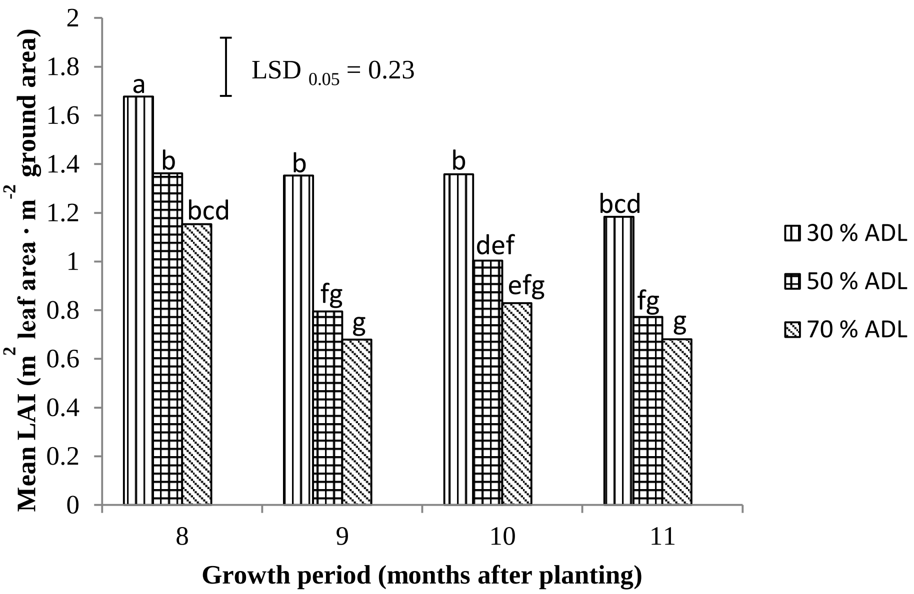

Figure 3.1. Leaf area index of *P. sidoides* in response to water treatment, over the growing period. LSD: least significant difference, ADL: allowable depletion level.

Plant height was also significantly reduced by water stress, as shown in Figure 3.2. The well watered control had a significantly higher plant height but there were no significant differences observed between the moderately and severely stressed treatments. Mabhaudhi *et al*. (2011) reported a marginal decrease in plant height of bambara landraces under rainfed conditions when compared to irrigated conditions. This could be due to the lower amount of rain received by crops under rainfed conditions. Alishah *et al*. (2006) found that in basil an increase in water stress levels resulted in a decrease in plant height. Similar results were also reported for *Jatropha curcas* (Hedayati *et al*., 2013).

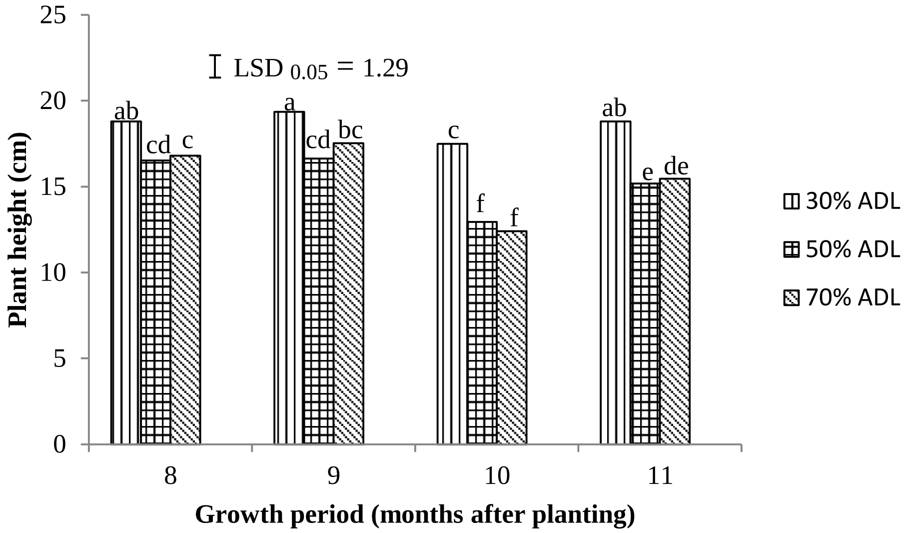

Figure 3.2. Plant height of *P. sidoides* over the growing period in response to water treatment. LSD: least significant difference, ADL: allowable depletion level.

Within the second month of water treatment application (8 months after planting - MAP) there were no significant differences in number of leaves across all the treatments (Figure 3.3). However after three and four months of water treatment application (9 and 10 MAP, respectively) the severely stressed treatment (70% ADL) had a significantly lower number of leaves, while there were no significant differences between the well watered control and the moderately stressed treatments. No significant differences were observed again at four months after water treatment application between all the treatments. The sudden increase in number of leaves, 11 month after planting, was due to the fact that the data was taken a week after irrigation of all water treatments, resulting in plant recovery. Since most of these new leaves were small, the mean LAI did not increase (Figure 3.1). Significant reductions in the number of leaves due to water stress were also reported in other crops such as parsley (Petropoulos *et al*., 2008), basil (Alishah *et al*., 2006) and common beans (Ghanbari *et al*., 2013). According to Munné-Bosch and Alegre (2004) leaf senescence is an adaptive strategy that contributes to plant survival under stress, including water stress. Decreasing of the canopy leaf area through reduced growth, is also another strategy to minimize water loss under water stress conditions

(Chaves *et al*., 2003). Leaf senescence may be sequential, starting gradually from oldest to youngest leaves, and depending on the duration and severity of stress, may allow young leaves to grow once stressful conditions have passed (Munné-Bosch and Alegre, 2004). A decrease in total leaf surface due to water stress induced senescence leading to leaf abscission, was reported on *Cichorium intybus* (Vandoorne *et al*., 2012).

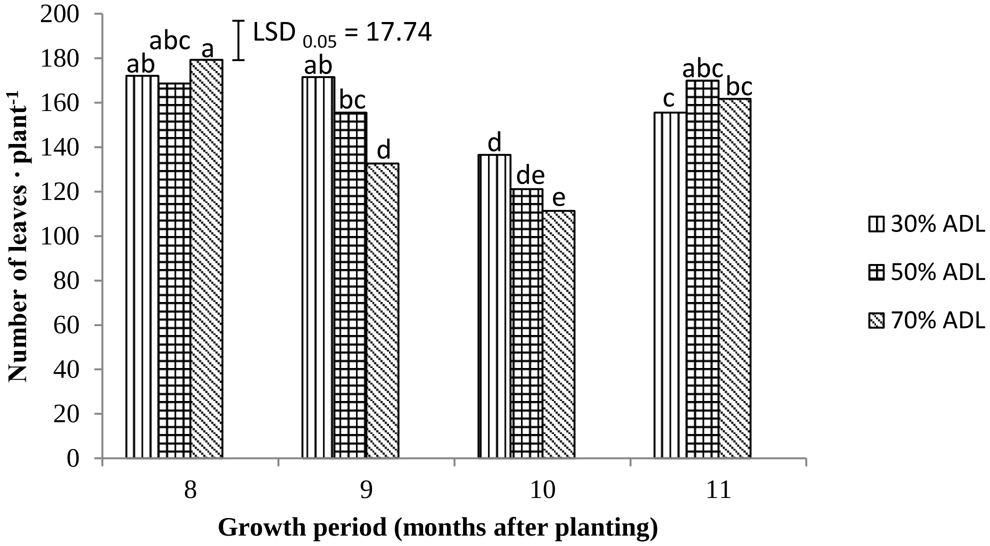

Figure 3.3. Number of leaves of *P. sidoides* over the growing period, in response to water treatment. LSD: least significant difference, ADL: allowable depletion level.

Water treatment also had a significant effect on leaf area, as indicated in Table 3.5. There was a declining trend in leaf area with an increase in water stress. The well watered control had a significantly higher leaf area compared to the severely stressed treatment. The moderately stressed treatment showed no significant difference in leaf area when compared to both the well watered control and severely stressed treatment, respectively. Similar results were reported by Liu *et al*. (2006) on potatoes, where the full irrigation treatment (irrigating to compensate for the full evapotranspiration water loss) had a significantly higher leaf area compared to the deficit irrigation and partial root drying treatments, with no significant differences observed between the two stress treatments. Karkanis *et al*., 2011, found the lowest leaf area of velvetleaf plant (*Abutilon theophrasti*) for the water stressed treatment (50% refill of field capacity refill), with the highest leaf area in the well watered control (100% field capacity refill). In another study on vegetable amaranth, Liu and Stützel (2002) reported a significantly lower leaf area for water stressed plants, where irrigation was withheld for a certain period, compared to the control which was irrigated to 90% of the water holding capacity of the soil, for all genotypes studied.

Table 3.5. Average leaf area index, leaf area per plant and stomatal conductance of *P. sidoides*, in response to water treatment.

Treatments ADL (%)	Mean LAI m^2 leaf area · m^{-2} ground area	Mean leaf area cm^2 · $plant^{-1}$	Mean conductance mmol m^{-2} s^{-1}
30	1.328^{a}	899.6^{a}	100.5^{a}
50	1.009^{b}	707.9^{ab}	49.04^{b}
70	0.934^{b}	617.3^{b}	36.44^{c}
$LSD_{0.05}$	0.11	204	9.99

*LSD: least significant difference, ADL: allowable depletion level.

Although nitrogen application had no significant effect on the other parameters, it had an effect on number of leaves at harvest. Figure 3.4 shows that nitrogen at the rate of 100 kg · N · ha^{-1} had a significantly higher number of leaves than the other treatments. Hussain *et al*. (2006) reported that the maximum number of branches per plant was found with the application of 90 kg · N · ha^{-1}, on asparagus. Zhu *et al*. (2009) found that the application of medium amounts of N and P fertilizer on *Bupleuri radix*, either alone or in combination, increased shoot growth amongst other parameters. Araya *et al*. (2006) reported that application of organic N at the rate of 100 kg · ha^{-1} increased fresh herbage and oil yield of rose scented geranium, compared to the control in the first harvest. In their second harvest both inorganic and organic N at the rate of 100 kg · ha^{-1} increased fresh herbage and oil yield over the control (Araya *et al*., 2006).

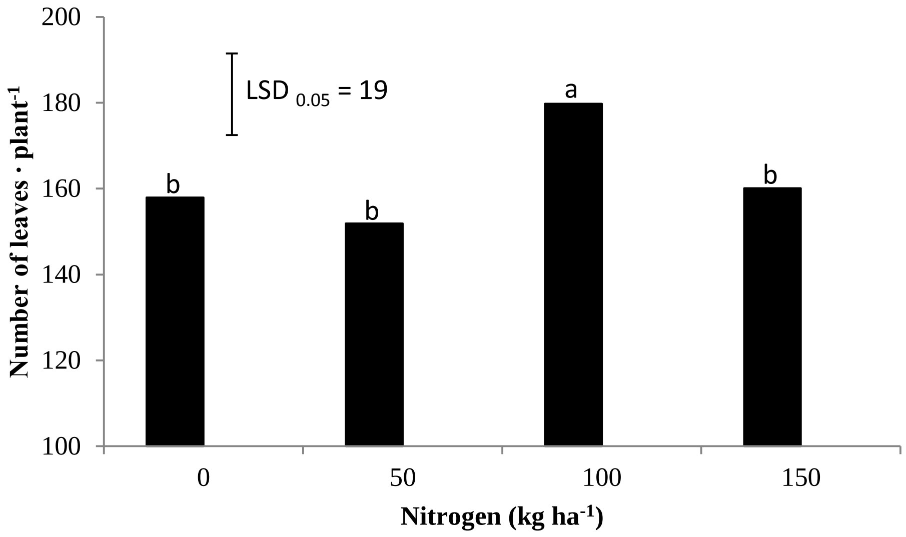

Figure 3.4. Average number of leaves per plant, of *P. sidoides*, in response to nitrogen level, at harvesting. LSD: least significant difference.

3.3.2. Stomatal conductance

The average stomatal conductance of *P. sidoides* as affected by water stress at different times over the growth period is shown in Figure 3.5. Stomatal conductance of the well watered control (30% ADL) was always significantly higher than that of the stressed treatments, while the 50% and 70% ADL treatments did not differ significantly from each other in most cases. Nitrogen did not show a significant effect on stomatal conductance. Green and Mitchell (1992) also reported a lack of N-related differences in stomatal response to water stress of loblolly pine seedlings.

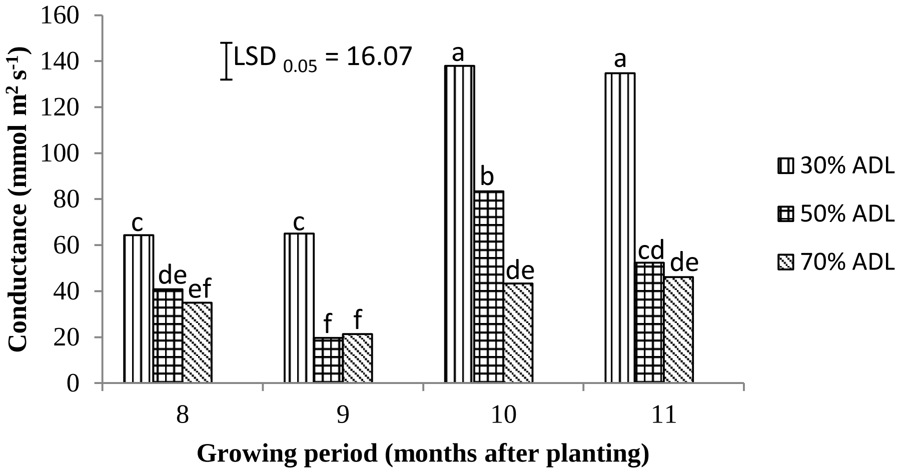

Figure 3.5. Average stomatal conductance of *P. sidoides* at different times over the growing period, in response to water treatments**.** LSD**:** least significant difference, ADL: allowable depletion level.

The stomatal conductance results, when observed across water stress treatments (Table 3.5), showed that it decreased with an increase in the stress. The well watered control had a significantly higher stomatal conductance compared to the other two water treatments. The moderately stressed treatment (50% ADL) also had a significantly higher stomatal conductance than the severely stressed treatment (70% ADL). The results on stomatal conductance in this study are consistent with results of work done on other crops. Eiasu *et al.* (2012) found that rose-scented geranium plants (*Pelargonium capitatum*) exposed to water stress had a lower stomatal conductance compared to those irrigated more often. Karkanis *et al.* (2011) reported that water stress reduced stomatal conductance of velvetleaf by 37 – 89%. All the species studied by Galméz *et al*. (2007) showed a progressive decline in stomatal conductance as water stress intensified.

The increase in stomatal conductance observed in month 10 and 11, could have been due to a decrease in vapor pressure deficits (VPD) in March and April 2013 (Table 3.3). Increases in VPD between leaf and air results in partial closure of the stomata, thus decreasing stomatal conductance so as to prevent excessive dehydration and physiological damage (Oren *et al.*, 1999, Ocheltree *et al.*, 2014). Sweet pepper plants grown under low VPD consistently maintained a higher stomatal conductance compared to plants grown at ambient and high level

VPD (Zabri and Burrage, 1998). Similar results were reported by Comstock and Ehleringer (1993) in their study on common beans; and Dai *et al*. (1992) on castor bean.

The higher stomatal conductance observed for the well watered control was the result of fully open stomata on both the abaxial and adaxial side of the leaves (Figure 3.6.A and B). The stomata on the moderately stressed samples were opened on the abaxial side and partially closed on the adaxial side of the leaf sample. The lowest stomatal conductance of the severely stressed plants was due to the stomata that were partially closed on the abaxial side, to fully closed on the adaxial side, as was observed under the electron microscope.

Although stomatal regulation in response to water stress has been a controversial issue for long, it has been recognized that stomatal closure results in a limiting conductance, controlling the flow of water through the plant (Comstock and Mencuccini, 1998). The mentioned study suggested a simple threshold model where stomatal closure is triggered as leaf water potential reaches a critical stress level. Stomatal closure is thus amongst the earliest responses to water stress, protecting the plants from extensive water loss (Chaves *et al*., 2003).

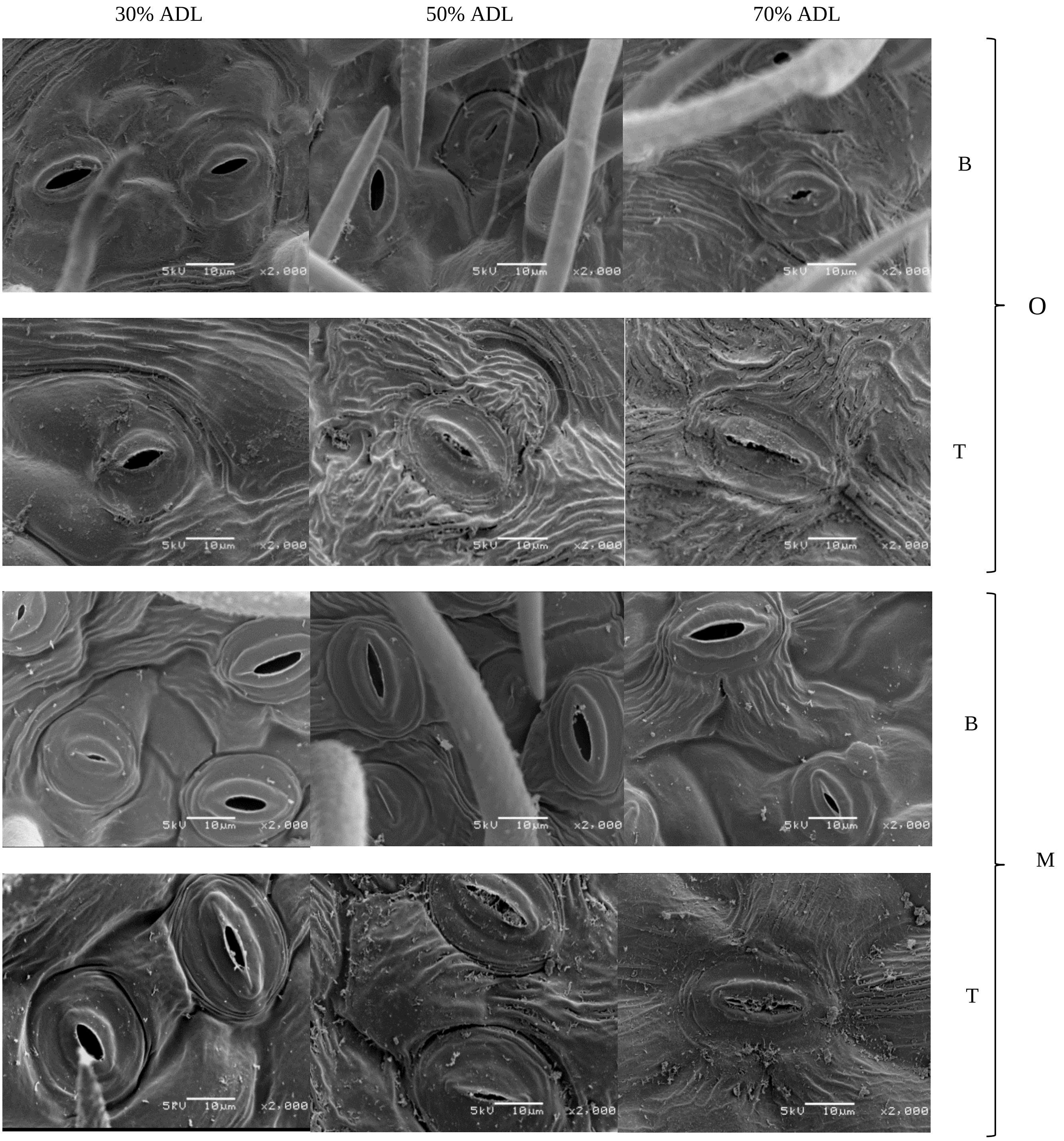

Figure 3.6.A. *P. sidoides* leaf samples showing stomatal pores as observed under an electron microscope. O = old leaves, M = mature leaves and Y = young leaves. B = abaxial side and T = adaxial side

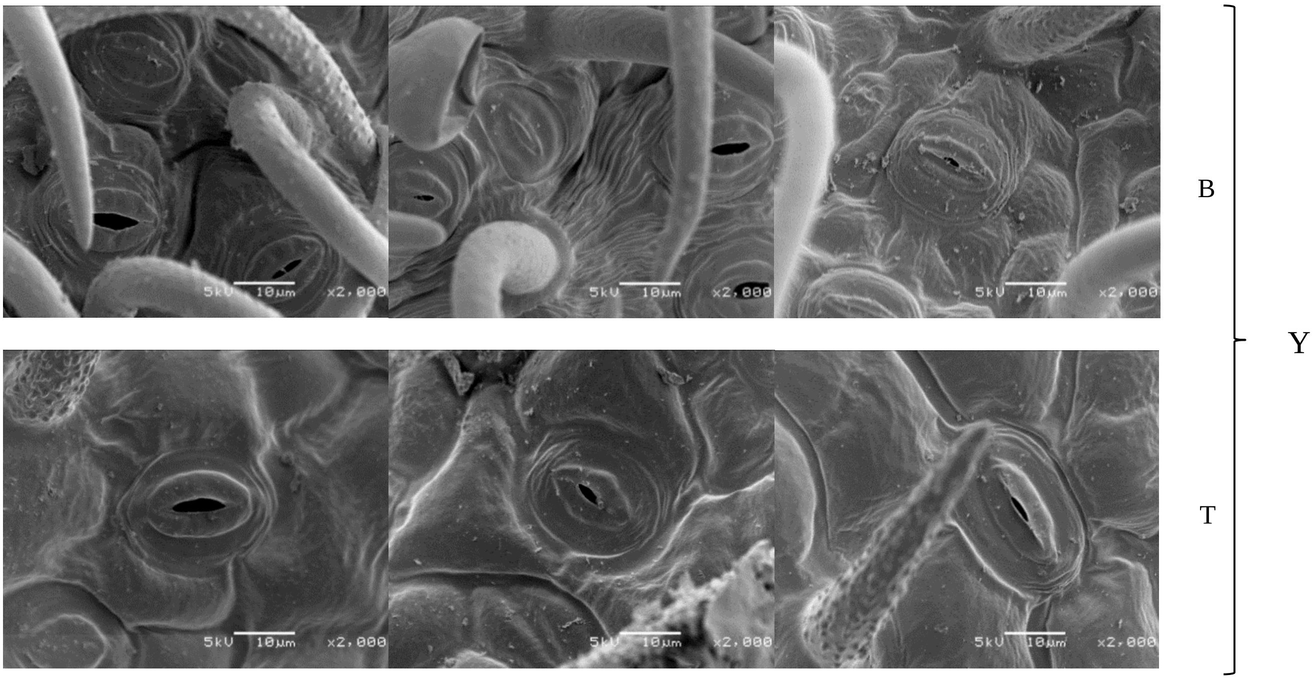

Figure 3.6.B. *P. sidoides* leaf samples showing stomatal pores as observed under an electron microscope. O = old leaves, M = mature leaves and Y = young leaves. B = abaxial side and T = adaxial side.

3.4. Conclusions

There was no significant interaction effect between nitrogen and water level for all the parameters measured in this study. Water stress significantly decreased stomatal conductance. Closing of the stomata is a physiological mechanism employed by plants to cope with water stress. However because stomata are the pathway for both water and CO_2 exchange with the atmosphere, this mechanism has a negative effect on photosynthesis, and therefore on plant growth. Microscopic observations confirmed that *P. sidoides*, like most other plant species, respond to water stress by closing their stomata. It was also observed that water stress resulted in closing of the stomata on the adaxial side of the leaves first, followed by closing of those on the abaxial side.

Morphologically, plants respond to water stress by leaf senescence, smaller canopy and smaller leaves, amongst others. *P. sidoides* showed similar response with reductions in LAI, plant height and leaf area per plant. These observed morphological responses probably resulted from reduced photosynthetic rate, since CO_2 uptake was decreased by closing of the stomata under stress conditions. Nitrogen level had a significant effect on number of leaves per plant, but not on leaf area, which means that although more leaves were stimulated, they were not bigger in size and therefore did not result in higher LAI. The study presented the first report on response of *P. sidoides* to water stress and nitrogen levels, which could be important in the establishment of nitrogen and water management guidelines for cultivation of *P. sidoides*.

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CHAPTER 4

YIELD RESPONSE OF *PELARGONIUM SIDOIDES* DC. TO WATER AND NITROGEN MANAGEMENT

Abstract

Pelargonium sidoides is an important traditional medicine in South Africa whose successful commercialization has resulted in intensified harvesting of wild populations. Information on cultivation practices such as nitrogen and irrigation requirements of *P. sidoides* is limited or non-existent. The aim of the study was to investigate the effect of water and nitrogen level on yield components of *P. sidoides*. The study was conducted under a rainshelter as a randomized complete block design with three irrigation levels, four nitrogen levels and three replicates. The irrigation treatments were 30% allowable depletion level (ADL) (well watered treatment), 50% ADL (moderately stressed treatment) and 70% ADL (severely stressed treatment), while the nitrogen levels were 0, 50, 100 and 150 $kg \cdot N \cdot ha^{-1}$. Nitrogen and water levels had no significant interaction effect on measured parameters. The well watered treatment had a significantly higher total biomass, fresh and dry root yield and nitrogen use efficiency compared to the water stressed treatments. Water use efficiency was significantly lower under well watered conditions. Water stress significantly increased nitrogen content and chlorophyll content of *P. sidoides* plants and leaves, respectively. Nitrogen levels of 50 and 100 $kg \cdot N \cdot ha^{-1}$ resulted in a significantly higher total biomass compared to the control. Plant nitrogen content was increased by the application of nitrogen but the nitrogen use efficiency was significantly reduced by nitrogen application. The study further showed that irrigation can increase root yield of *P. sidoides* significantly while N levels did not have a significant effect on root yield. The increase in total biomass with increased N levels in this study, could be an unnecessary expense as it did not translate into an economic benefit. This could have been due to almost sufficient residual N in the soil and the results could be different in a very poor soil. This report provides important information on irrigation and nitrogen management for growers; and could have a significant impact on how farmers manage conditions for cultivation of *P. sidoides*.

Keywords: water stress, nitrogen, allowable depletion level, medicinal plant

4.1. Introduction

Agriculture is the major user of water resources in many regions of the world. With increasing aridity and a growing world population, water will become an even scarcer commodity with significant uncertainty of supply and quality, in the near future (Pereira *et al*., 2002; Chaves *et al*., 2003; Fereres and Soriano, 2007). Understanding the behaviour of every crop to the amount of water applied is necessary to determine the effect that lack or excess of water may have on production, thus enabling appropriate irrigation management (Bilibio *et al*., 2011).

Irrigation, a major contributor to agricultural productivity, can increase land productivity by about 127% (Eiasu *et al*., 2009). In a drought prone country like South Africa, a shift in the allocation of water resources to non-agricultural economic sectors due to competition of use, may impose restrictions on agricultural business unless innovative irrigation management for each crop is introduced (Eiasu *et al*., 2009). Irrigation water management will have to be carried out more efficiently in future, with the aim of saving water while maximising productivity (Fereres and Soriano, 2007).

Deficit irrigation is becoming an important strategy to reduce agricultural water use in arid and semi-arid regions. It involves deliberate under irrigation of crops or irrigating below evapotranspiration requirements of a crop, to reduce water consumption while minimizing adverse effects of extreme water stress on yield (Pereira *et al*., 2002; Fereres and Soriano, 2007; Ayana, 2011). This practice requires good knowledge of the response of different crops to water deficits. Deficit irrigation does not always decrease yield and properly applied deficit irrigation may even increase crop yield (Bilibio *et al*, 2011). Fereres and Soriano (2007) mentioned that information on the effect of deficit irrigation on root crops is limited, given the difficulties in quantifying root biomass under field conditions.

Water use efficiency (WUE), which is explained biologically as carbohydrates formed through photosynthesis from CO_2, sunlight and water per unit of transpiration, can be simply described as crop yield per unit of water use (Howell, 2001). It can be generally defined as the ratio of crop yield to water used to produce the yield (Zhang *et al*., 2004), simply put as yield divided by water-use (Blum, 2009). Water-use is the total amount of water applied to grow the crop during the season and it includes transpiration, evaporation, run-off and deep drainage (Hunt and Kirkegaard, 2009).

Any stress involving nitrogen deficiencies and water shortages adversely affects the chlorophyll content of plants (Schlemmer *et al*., 2005). The utilization of nitrogen is coupled to that of water reaching the root zone, since plants take up inorganic nitrogen contained in water absorbed from the soil solution through their root system (Alva *et al*., 2006). Under deficient water supply conditions, root growth, physiological activity of roots and shoot growth can be constrained, indirectly leading to N-deficiency (Aujla *et al*., 2007). Restriction of plant development by low levels of external factors such as water, amongst others, causes the internal concentration of nitrogen to rise (Barker and Bryson, 2007). Leaching and plant uptake of nitrogen are therefore dependent on the available soil water, as the transport medium (Matzner and Richards, 1996; Everard *et al*., 2010). High irrigation levels have been reported to reduce NO_3 levels by leaching nitrogen out from the soil, while low irrigation levels provide insufficient water for N mineralization and for dissolving NO_3 (Abdel-Mawly, 2004).

Since nitrogen input is directly linked with irrigation, the most appropriate and cost effective management strategy would therefore be to integrate irrigation and nutrient inputs, especially nitrogen, as the two cannot be managed independently. Water stress tolerant plants growing in climates with seasonal water stress appear to maintain their root and nutrient uptake capacity during low water availability periods; and this may be very important for the continuation of plant growth as water availability declines (Matzner and Richards, 1996).

Over application of nitrogen fertilizers has caused many environmental pollution problems (Lee *et al*., 2011) and can lead to decreased yields due to luxury consumption (Alva *et al*., 2006). Nitrogen use efficiency (NUE) is defined as a proportion of all the nitrogen inputs that are removed in the harvested crop biomass, contained in recycled crop residues and incorporated into the soil organic matter and inorganic N pools (Cassman *et al*., 2002). Nitrogen source can have large effects on total nitrogen concentration in plants whereby plants grown on ammonium nutrition can have twice the nitrogen concentrations as plants grown on nitrate nutrition (Barker and Bryson, 2007).

Pelargonium sidoides is one of several geophytic species that are important traditional medicines in South Africa (Lewu *et al*., 2006). The tuberous roots of the species are the raw material for a phytomedicine used to treat acute bronchitis and infections of the upper respiratory tract. Over exploitation of the species, for local traditional use and international pharmaceutical companies has led to declines in wild populations. It has been reported that *P. sidoides* growing naturally under low rainfall conditions exhibited increased medicinal value, compared to the one growing in high rainfall conditions (White *et al*., 2008). However, little is known about cultivation of this species especially the effect of soil water and N levels on its growth and yield. The study was thus undertaken to determine the effect of water and nitrogen level on yield of *P. sidoides*.

4.2. Materials and methods

For details on site description, plant material, trial design and treatment application please refer to Chapter 3.

4.2.1. Data collection and statistical analysis

Leaf chlorophyll content was measured on a fully matured leaf, with a chlorophyll content meter (SPAD 502 plus, Konica Minolta, Japan) at harvesting. Three plants per plot which represented three replicates were used for this measurement. Total biomass and root yield were determined by weighing the whole plant first and then roots only, on a field scale (Platform digital scale, W113, Richter scale) after harvesting, where six plants per plot were measured. The roots were then oven dried (Economy oven, 620 digital, Labotec) at 70 °C until constant mass, to obtain dry root yield.

A plant sample from each treatment plot was then subjected to nitrogen content analysis by adapting established methods of Jimenez and Ladha (1993); and Matejovic (1995). The samples were finely milled into powder and approximately 8 to 14 mg per sample were weighed into a tin foil container for N determinations on a Carlo Erba NA 1500 Nitrogen/Carbon/Sulphur Analyzer. A dry oxidation method, known as the Dumas method was used, where the sample and tin container were ignited at high temperature (1020°C) in oxygen, on a chrome oxide catalyst, to produce carbon dioxide, nitrogen gas, oxides of nitrogen and other oxides. The gases were then passed through silvered cobalt oxide, then a column of copper at 540°C, which reduces the oxides of nitrogen to nitrogen gas, removing the excess free oxygen. After the removal of water vapour and carbon dioxide, the N_2 gas was 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 was calibrated using a pure organic compound of known composition and the compound chosen for our calibration standard was

the ethyl ester of 4-Aminobenzoic acid, which contains 8.48% N. PeakNet software (Dionex Corporation, 1998) with an external A/D interface (UI20 Universal interface, Dionex) was used for data collection, peak integration, calibration and computation of concentrations.
Plant nitrogen content was determined on dry plant material, thus the values are on a dry weight basis. Total N taken up per plot was determined using the formula:

Total N uptake = (Ng/X) x Y

Where Ng is nitrogen content (grams) of the plant sample, X is the mass of the plant (grams) and Y is the total mass of all the other plants from the same plot.

NUE was estimated as amount of dry matter produced per kg nitrogen applied (Fageria and Baligar, 2005). WUE was calculated according to Hunt and Kirkegaard (2009) and expressed as $g \cdot mm^{-1}$. In this study the roots are the harvested parts and thus root yield, on a dry mass basis, was used in both the calculations and therefore both are expressed as NUE_{root} and WUE_{root}.

Data were subjected to analysis of variance (ANOVA) using *GenStat*® version 11.1. Treatment means were separated using Fisher's protected T-test for least significant differences (LSD) at 5% level of significance.

4.3. Results and discussion

4.3.1. Soil water deficit and water use

Figure 4.1 shows the soil water deficits allowed, per water treatment, before irrigation was initiated. The amount of water applied over the treatment period was 466 mm for the well watered treatment (30% ADL), 307 mm for the moderately stressed treatment (50% ADL) and 256 mm for the severely stressed treatment (70% ADL). The total relative evapotranspirative demand (ET_0) for the treatment period was 663.3 mm (see Table 3.2). The well watered treatment in this study recorded the highest water usage which is in agreement with research done by Eiasu *et al*. (2009) on rose-scented geranium. Darwish *et al*. (2006) also recorded the highest water usage, of 510.6 mm, for the well watered control (irrigating to 100% ET), followed by 407.3 mm for the moderate deficit irrigation (irrigating to 80% ET) and 313.6 mm for the severe deficit irrigation (irrigating to 60% ET).

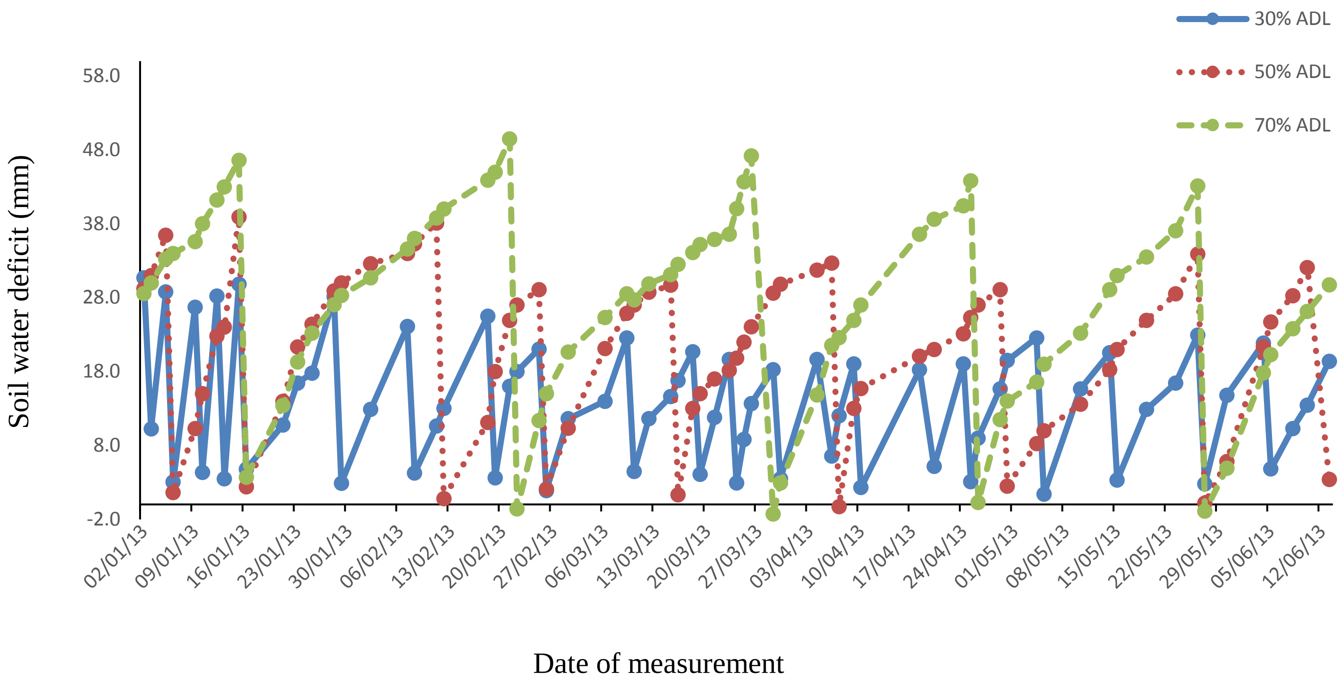

Figure 4.1. Changes in soil water deficits (top 400 mm root zone) for the well watered (30% ADL), moderately stressed (50% ADL) and severely stressed (70% ADL) treatments.

4.3.2. Total fresh biomass yield

There was no significant interaction between nitrogen and water stress level for all parameters measured. N rate had a significant effect on total biomass where there was an increase in total biomass from 0 to 50 kg · N · ha^{-1} (Figure 4.2.). Thereafter a slight decrease was observed at 100 and again at 150 kg · N · ha^{-1}, although it was not significantly different from total biomass produced with the 50 kg · N · ha^{-1}. Hussain *et al*. (2006) reported that a nitrogen rate of 90 kg · N · ha^{-1} increased plant weight of asparagus and attributed this increase to an increase in number of branches and plant height, which enhanced photosynthetic activity. The highest total biomass for palmarosa, an essential oil crop, was produced by applying 80 kg · N · ha^{-1} under rainfed conditions (Rao, 2001). A rate of 160 kg · N · ha^{-1} significantly increased herbage yield of *P. graveolens*, beyond which there were no significant yield improvements (Ram *et al*., 2003). Araya *et al*. (2006) found an increase in fresh herbage yield over the control at 100 kg · N · ha^{-1} beyond which there were no significant increases in yield for rose-scented geranium (*Pelargonium capitatum*).

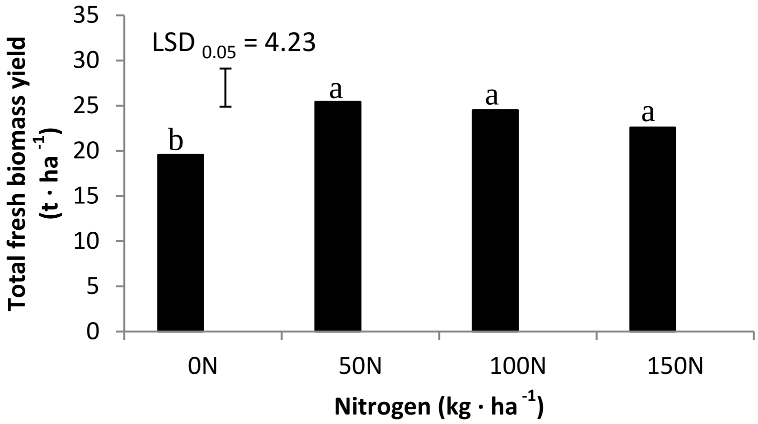

Figure 4.2. Total fresh biomass yield of *P. sidoides* in response to nitrogen level.

Water treatments also had a significant effect on total fresh biomass yield. Figure 4.3 shows that the well watered treatment had a significantly higher total biomass yield than the two water stressed treatments. There was no significant difference between the moderately and severely stressed treatments. Similar results were reported previously on other crops. Eiasu *et al*. (2009) reported that the well watered treatment, referred to as lower maximum allowable depletion (MAD) level in that study, resulted in better herbage yield of rose-scented geranium. Karkanis *et al*. (2011) also found that velvetleaf plants produced the lowest aboveground biomass under water stress. Moderate water stress, at 50 % MAD, and severe water stress, at 80% MAD, resulted in significant decreases in above ground fresh yield of *Thymus daenensis*, when compared to the non- stressed control (20% MAD) (Bahreininejad *et al*., 2013). The same study found no significant differences between the moderate and severe water stress treatment on above ground fresh yield, in the second year.

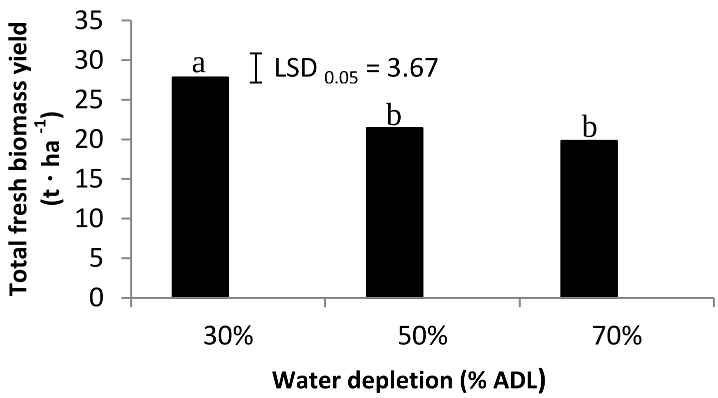

Figure 4.3. The effect of soil water level on total fresh biomass yield of *P. sidoides*.

In the first year of harvesting *P. sidoides* from the wild, Lewu *et al*. (2007) found a fresh biomass yield of 5.7 kg · plot^{-1} of 20 m^2 with a mean population of 23.6 plants, which translates to 11 800 plants · ha^{-1}. This yields to about 0.24 kg · plant^{-1} or 2.85 t · ha^{-1}. In our study the severe water stress treatment (70% ADL) resulted in a similar yield of 0.24 kg · plant^{-1}. However, the increase in plant population from 11 800 plants · ha^{-1} as reported in the literature by Lewu *et al*. (2007) to 66 666 plants · ha^{-1} in our study, increased yield from 2.85 to 16 t · ha^{-1} under water stressed conditions. Although the growth period of the wild population was not determined by Lewu *et al*. (2007), the current study shows that irrigation can increase total biomass yield to more than 26 t · ha^{-1} when compared to only 2.85 t · ha^{-1} as reported in the literature under rainfed conditions in the wild.

4.3.3. Root yield

Fresh root yield and dry root yield, respectively, followed the same trend as biomass yield where water stress significantly reduced the yield (Table 4.3), while N application had no significant effect on root yield. The well watered treatment resulted in significantly higher values than the water stressed treatments for both fresh and dry root yield, with no significant differences between the water stressed treatments. Plants exposed to moderate water stress had a significantly lower dry matter content compared to the well watered treatment (Table 4.3). Other researchers reported similar results in different crops. Vandoorne *et al*. (2012), in their study on root chicory, found that the mean root fresh yield was lowered by water stress, with a decrease of more than 50%, compared to the control. Fresh root yield of plain leafed and turnip rooted parsley exposed to a higher water stress level was significantly decreased in both years of the study (Petropoulos *et al*., 2008). Darwish *et al*. (2006) reported that severe deficit irrigation (60% ET) led to a 21% loss in potato fresh yield, due to lowered tuber dry matter production and average mass of the commercial tubers. The dry matter yield of whole plants and individual parts of *Chenopodium quinoa* plants was significantly higher in the control than under drought conditions (Gonzàlez *et al*., 2009). In their study, Bahreininejad *et al*. (2013), found that the dry root yield of *Thymus daenensis* was significantly higher in the non-stressed control (20% MAD) compared to the moderate and severe water stress treatments (50% and 80% MAD, respectively), while there were no significant differences between the two water stress treatments in the first year. The low prices (R1.60 – R4.50 · kg^{-1}) fetched by harvesters for wet mass of *P. sidoides* roots as compared to what the middleman is paid (R48 – R120 · kg^{-1}) (Motjotji, 2011) could be due to the initial high moisture content in the roots, in addition to marketing costs. If the harvesters were to dry the plant material themselves, they would possibly fetch a maximum price of R18.00 · kg^{-1}, given the lower moisture content, but this means the middleman still makes a major profit.

Table 4.1. Fresh, dry root yield and total biomass dry matter content (percentage) of *P. sidoides* in response to water treatment.

Water depletion (% *ADL)	Fresh root yield (t · ha^{-1})	Dry root yield (t · ha^{-1})	Average dry matter content (%)
30	19.77 ^{a}	3.58 ^{a}	27.24 ^{a}
50	14.52 ^{b}	2.51 ^{b}	24.58 ^{b}
70	14.05 ^{b}	2.15 ^{b}	25.57 ^{ab}
$LSD_{0.05}$	2.81	0.59	1.93

* LSD = least significant difference, ADL = Allowable depletion level

4.3.4. Chlorophyll and nitrogen content

Water stress significantly increased chlorophyll content as shown in Figure 4.4. There was a significant increase from the well watered treatment (30% ADL) to the moderately stressed treatment (50% ADL); however there was no significant difference between the two stress treatments. The nitrogen content (%) in the leaves and in the whole plant (Table 4.4) followed a similar trend, where both the moderately stressed and severely stressed treatments (70% ADL) were not significantly different from each, other but were significantly higher than the well watered treatment. Our results are consistent with findings on other crops. Gonzàlez *et al*. (2009) reported no significant differences in chlorophyll content of *Chenopodium quinoa* plants, between the control and drought treatments, but the leaf nitrogen content was significantly higher in the drought treatment compared to the control. Rahimi *et al*. (2010) also reported that drought stress increased chlorophyll content significantly in *Plantago ovata* and *P. psyllium* plants. A decrease in relative water content and leaf water potential, due to water stress, resulted in increased leaf chlorophyll content, by 17 and 37%, for *P. ovata* and *P. psyllium*, respectively (Rahimi *et al*., 2010).

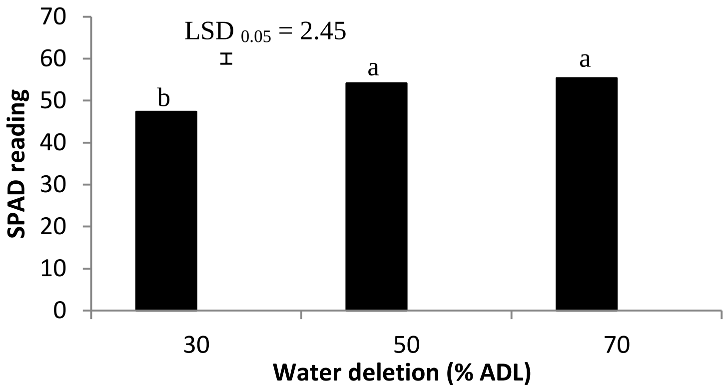

Figure 4.4. Chlorophyll content (SPAD) of *P. sidoides* as affected by soil water level.

Table 4.2. Nitrogen content (%) in the leaves and in whole *P. sidoides* plant in response to water treatments.

Water depletion (% *ADL)	N content (leaves)	N content (whole plant)
	%	
30	2.69 [b]	1.50 [b]
50	3.17 [a]	2.16 [a]
70	3.21 [a]	2.26 [a]
$LSD_{0.05}$	0.19	0.24

*LSD = least significant difference, ADL = Allowable depletion level

An increase in nitrogen rate (kg · ha^{-1}) resulted in a significant increase in nitrogen content (%) of the whole plant (Figure 4.5). The rate of 150 kg · N · ha^{-1} resulted in a significantly higher nitrogen content than the control and lowest application. The control, 50 and 100 kg · N · ha^{-1} did not differ significantly from each other, while the 100 kg · N · ha^{-1} did also not differ significantly from the 150 kg · N · ha^{-1} treatment. Similarly, nitrogen application significantly affected nitrogen concentration of potato tubers, where application of N at the rate of 240 -250 kg · ha^{-1} resulted in significantly higher tuber N concentration (Millard, 1986). Millard and MacKerron (1986) also reported that N application increased nitrogen concentration in leaves and stems in potatoes. The highest nitrogen content for isabgol (*Plantago ovata*) under medium and low water stress (60% and 80% of FC, respectively) was observed with the highest application of nitrogen at the rate of 180 mg · N · kg^{-1} soil in pots (Rahimi *et al*., 2013).

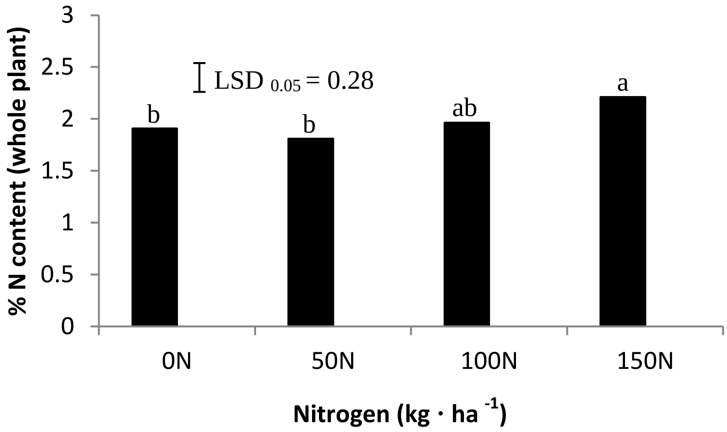

Figure 4.5. Nitrogen content, on a dry mass basis, of whole plants of *P. sidoides* as affected by nitrogen level.

4.3.5. Nitrogen uptake and Nitrogen use efficiency (NUE_{root})

The two water stress treatments, moderate (50% ADL) and severe water stress (70% ADL), showed a significantly higher total nitrogen uptake compared to the well watered treatment, as indicated by Figure 4.6. The results are consistent with findings of Scagel *et al*. (2011) on *Rhododenron*, where they reported that a decrease in water stress was associated with lower N uptake. *Panicum maximum* plants undergoing water stress showed an increase in nitrogen concentration (Dias-Filho *et al*., 1992). Water stress tolerant growing under seasonal water are reported to have the capability of maintaining nutrient uptake during times of low water availability (Matzner and Richards, 1996). Plants are known to increase their root depth under drought conditions so as to extract water from wet deeper soil layer. Several species have shown the ability to extract nutrients from shallow dry soil layers as long as their root system can access deeper wet layers (Matzner and Richards, 1996).

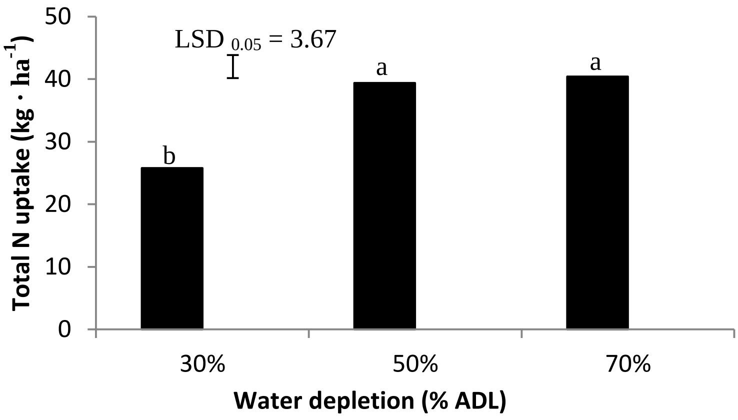

Figure 4.6. Total nitrogen uptake, on a dry mass basis, of *P. sidoides* plants exposed to different water levels.

Nitrogen level again had a significant effect on nitrogen use efficiency (NUE_{root}) where an increase in the application rate resulted in a decrease in NUE_{root} (Figure 4.7). The 50 kg · N · ha^{-1} treatment had significantly higher NUE_{root} than the 100 and 150 kg · N · ha^{-1} treatment. Badr *et al*. (2012) also found that an increase in N application rate resulted in a decrease in NUE of potato tubers. Similar results were reported by Colla *et al*. (2005), Fandika *et al*. (2010) and Ospina *et al*. (2014), on potatoes. Increases of nitrogen level from 80, 160 to 200 kg N · ha^{-1} resulted in significant decreases in NUE of potato tubers, from 26.81 to 13.32 kg yield · kg^{-1} N (Jamaati-e-Somarin *et al*., 2010).

Water stress also had an effect on NUE_{root} where it was observed that the well watered treatment had a significantly higher NUE_{root} than the two water stress treatments (Figure 4.8). Abdel-Mawly (2004) reported a marked increase in recovery of applied N with an increase in the supply of irrigation water on carrots. Darwish *et al*. (2006) attributed the lower NUE observed under moderate and severe deficit irrigation in potatoes, to a lower tuber dry matter yield under those conditions. Irrigation treatment had a significant effect NUE of potatoes where the highest value of 176 kg yield · kg^{-1} N and lowest values of 55 kg yield · kg^{-1} N, were reported for the well irrigated treatment and severe water deficit treatment, respectively (Badr *et al*. 2012). Irrigation increased NUE of four potato cultivars studied by Fandika *et al*. (2010), by 29.6, 26.2, 60 and 104.2%, respectively.

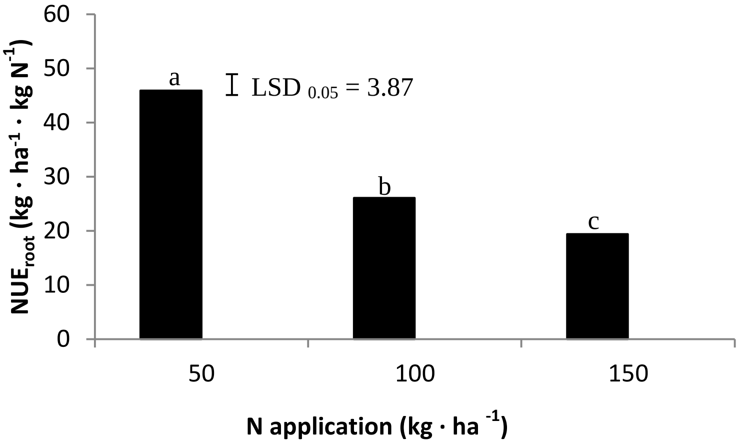

Figure 4.7. Nitrogen use efficiency of *P. sidoides* plants, on a dry root mass basis, as influenced by nitrogen level.

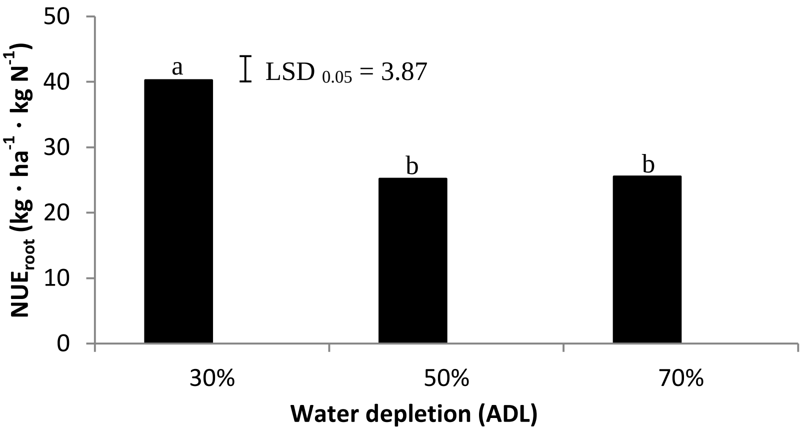

Figure 4.8. Nitrogen use efficiency of *P. sidoides* plants, on a dry root mass basis, as influenced by soil water level.

4.3.6. Water use efficiency (WUE_{root})

According to Figure 4.9, the well watered treatment showed significantly lower water use efficiency compared to the water stress treatments, which were not significantly different from each other. A decrease in WUE_{root}, averaging 1.4 g DW · kg^{-1} H_20, in two varieties of sugarbeet under well watered conditions was reported by Rytter (2005). Irrigation water productivity, which is the final yield per unit of applied water, of potato tubers was increased under moderate water stress (Darwish *et al.*, 2006). Lakew *et al.* (2014) studied, on onion, the total water use efficiency (TWUE), which is the onion yield per mm^{-1} of total water applied and net water use efficiency (NWUE) is the yield produced per mm^{-1} of water consumed. The results indicated that under full irrigation level (FIL, 100% ET_0) both TWUE and NWUE were significantly decreased, as compared to the mid irrigation level (MIL, 75% ET_0) and half irrigation level (HIL, 50% ET_0) (Lakew *et al.*, 2014). The approximate values for TWUE for FIL was 15.45 kg/mm, for the MIL it was 22.41 kg/mm and for the HIL it was 38.24 kg/mm; while for the NWUE they were 16.85 kg/mm for FIL, 28.81 kg/mm for MIL and 40.02 kg/mm for HIL (Lakew *et al.*, 2014). Eiasu *et al*. (2012) reported an increase in WUE with an increase in irrigation frequency for rose-scented geranium (*Pelargonium* spp) and this could be attributed to low biomass production under drought conditions (Wu *et al*., 2008). The difference could be that in their study, Eiasu *et al*. (2012), the harvested part was leaves while in the current study it was roots.

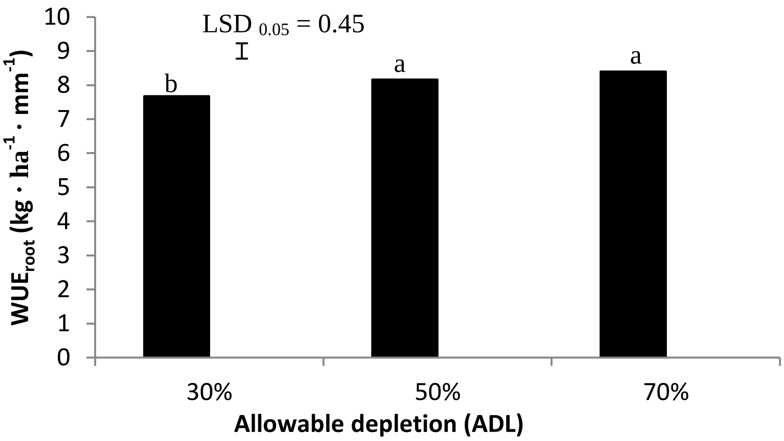

Figure 4.9. Water use efficiency of *P. sidoides*, on dry root mass basis, in response to different soil water levels.

4.4. Conclusions

The well watered treatment (30% ADL) used more water than the moderate (50% ADL) and severe (70% ADL) treatments and that could be attributed to larger canopies and increased water availability. An increase in water stress resulted in an increase in nitrogen uptake, nitrogen content and chlorophyll content. However, the total biomass and root yield was decreased by water stress. This could be attributed to growth inhibition under water stress, to save and redistribute limited resources, which is counter-productive in terms of yield. WUE, based on dry root mass, was increased by water stress meaning that more root mass was produced per mm^{-1} of irrigation water applied. The total biomass yield increased with increasing nitrogen level until the rate of 100 kg · N · ha^{-1}, while the nitrogen content of whole plant continued to increase with further increases in nitrogen level. The increased N content, of both the leaves and whole plant, under water stress could be due to the accumulation of N in the plants, but it may not have been utilized for plant development due to water stress, resulting in a low NUE_{root} under the same stress condition. The study showed that irrigation can increase root yield of *P. sidoides* significantly, while N levels did not have a significant effect on root yield, although an increase in total biomass was observed. The increase in total biomass at 100 kg · N · ha^{-1} did not relate into an increase in root yield and is not important in cultivation of *P. sidoides* since the roots are used for medicinal purposes. The increased total biomass, in this study, is therefore an unnecessary expense as it did not translate into an economic benefit for the farmer. The major profit differences between harvesters and the middleman could mean that the growers or harvesters can be advised to semi-process and supply directly to the market.

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CHAPTER 5

CHEMICAL COMPOSITION OF *P. SIDOIDES* DC. AS AFFECTED BY SOIL WATER AND NITROGEN LEVEL

Abstract

Many South African medicinal plants, including *Pelargonium sidoides*, are used in the production of internationally marketed herbal remedies for the treatment of various ailments. The combined traditional and commercial use of *P. sidoides* has led to the extensive harvest of wild plants in the Eastern Cape and Free State provinces of South Africa and in Lesotho. Currently no relationship has been established between the chemical composition of *P. sidoides* and production practices such as water and nitrogen supply. The aim of the study was to investigate the effect of soil water and nitrogen levels on chemical composition of *P. sidoides*. A trial was conducted under a rainshelter, with three predetermined irrigation levels and four different nitrogen levels. The soil profile was irrigated back to field capacity when 30%, 50% and 70% of plant available water was depleted. Nitrogen was applied at rates of 50, 100 and 150 $kg \cdot N \cdot ha^{-1}$ with a control of no (0) nitrogen. Dried root samples were analyzed for the presence of two standard compounds, scopoletin and esculin, using thin layer chromatography (TLC) and for metabolite profiling using the nuclear magnetic resonance technique (NMR). TLC analysis showed the presence of the two standard compounds in all treatment samples analyzed. The orthogonal partial least square discriminatory analysis (OPLS-DA), which was performed on the NMR spectral data, showed separation between the irrigation treatments, resulting in two clusters representing the well watered treatment and the water stressed treatments. Asparagine, arginine, sucrose, xylose, glucose and citric acid were found to be the compounds associated with the separation. There was no separation of the samples regarding the nitrogen treatments which is indicative of the small effect of nitrogen on the metabolite content of the treatments. The study successfully differentiated the metabolites responsible for variation between well watered and water stressed root samples of *P. sidoides*. Scopoletin and esculin could not be identified from the NMR profiles as these compounds seemed to be present in lower concentrations than needed for NMR. This could possibly mean that *P. sidoides* plants need to be cultivated for two seasons or more for the water and nitrogen effects to be observed on the chemical composition, especially marker compounds.

Keywords: water stress, nitrogen, allowable depletion level, secondary metabolites, primary metabolites, NMR, metabolomic profiling.

5.1. Introduction

The performance of plants is greatly influenced by water deficits, in terms of the production and the formation of compounds; with the level and duration of stress being fundamental in the response of essential oil and medicinal plants (Alvarenga *et al*., 2011, Pirzad *et al*., 2012). Secondary compounds found in plants are associated with increasing survival, either by coping with unfavourable environmental conditions or by regulating some metabolic processes (Solecka, 1997). The yield and composition of secondary metabolites of certain medicinal plants vary within and between populations as influenced by geographic and environmental factors (White *et al*., 2008). Plant nutrients, especially nitrogen and water are the most important variables for producing a profitable crop (Schlemmer *et al*., 2005).

Commercial root extracts of *Pelargonium sidoides,* an important southern African medicinal plant, have become popular in many countries for the treatment of upper-respiratory tract infections (Seidel and Taylor, 2004, Wittschier *et al*., 2007, Agbabiak *et al*., 2008, White *et al*., 2008, Brendler, 2009). The phytochemical profiling of *P. sidoides* has indicated a dominating presence of polymeric pro-anthocyanidins and monomeric flavan-3-ols, besides considerable amounts of coumarins (Wittschier *et al*., 2007). Coumarins have been hypothesised to be produced in response to traumatic injury by plant diseases or during water stress (White *et al*., 2008, Ojala, 2001). They comprise a very large class of compounds found throughout the plant kingdom and although mainly synthesised in the leaves, they occur at the highest levels in the fruits, followed by the roots, stems and leaves (Ojala, 2001, Lacy and O'Kennedy, 2004, Jain and Joshi, 2012).

The medicinal properties of *P. sidoides* root extracts are ascribed to eight different coumarins, of which umckalin and 5, 6, 7 – methoxycoumarin are useful marker compounds as they appear to be absent in *P. reniforme* (Bladt and Wagner, 2007, Brendler and van Wyk, 2008, van Wyk, 2008; White *et al*., 2008; European medicines agency, 2012). Van Wyk (2008) reported the presence of gallic acids and methyls of gallic acids, flavonoids, flavan-3-ols and phytosterols in the roots. Mativandlela (2005) mentioned that two distinct coumarins; umckalin and its 7-*O*-methyl ester, together with four other methoxycoumarins and three unique coumarin sulphates were found in *P. sidoides* but not in *P. reniforme*. Some studies have suggested that various coumarin glycosides and coumarin sulphates are confined to *P. sidoides* (Brendler and van Wyk, 2008). The presence of esculin and scopoletin in the root extracts of *P. sidoides* has also been reported (Kayser and Kodziej, 1995, Gödecke *et al*., 2005, Kolodziej, 2007, European Pharmacopoeia 6.0, 2008). Esculetin and scopoletin are some of the most widespread coumarins in nature (Jain and Joshi, 2012). Scopoletin synthesis is post-infectionally activated in plants but can also be triggered by various abiotic stresses (Bourgaud *et al*., 2006). It also displays radical scavenging properties toward reactive oxygen species and may be involved in the reduction of oxidative stress in plant cells (Bourgaud *et al*., 2006). Umckalin may not be an active compound of *P. sidoides* but is used as a marker compound to differentiate between roots of *P. sidoides* and those of *P. reniforme,* as reported by Maree and Viljoen (2012). Although *P. reniforme* does not have umckalin, both the roots of *P. sidoides* and *P. reniforme* are traditionally used for the same purposes (Kolodziej, 2002, Mativandlela, 2005, White, 2006, White *et al*., 2008). *P. sidoides* has significantly higher yield of coumarins, with umckalin amounting for about 40% of total coumarin content (European medicines agency, 2012) and this could be a possible reason for preference of *P. sidoides* roots.

Metabolomics is an increasingly important OMICS method which comprises the detection of metabolites present within a biological system under specific conditions, thus providing a phenotypic assessment of the system (Tugizama *et al*., 2013). Biological important molecules have signals which are characterized by their frequency or chemical shift, intensity, structure and magnetic relaxation properties; all of which reflects the environment of the sample under detection (Krishana *et al.*, 2005).

The use of irrigation in cultivation of medicinal plants can increase the biomass yield and decrease chemical variability of plants, but due to the removal of natural environmental factors, it may reduce secondary metabolite production (White *et al*., 2008). Plants can sustain growth under drought conditions through osmoregulation which occurs via accumulation of non-reducing sugars, reducing sugars, organic acids or amino acids (Büssis and Heineke, 1998). Coumarins are synthesized in plant leaves and stored in the roots; therefore increasing leaf growth through irrigation and nitrogen application, may increase the concentration of chemical compounds in roots. This study was aimed at establishing whether there is any relationship between soil water, nitrogen and the chemical composition of *P. sidoides.*

5.2. Materials and methods

For a detailed description of the field trial please refer to Chapter 3.

5.2.1. Thin layer chromatography (TLC)

The European Pharmacopoeia 6.0 (2008) was adapted for the TLC (aluminum, $60f_{254}$) assay. Standard compounds, scopoletin and esculin, were acquired from Sigma Aldrich (South Africa). To prepare a reference solution 1 mg of scopoletin and 1 mg of esculin, respectively, were dissolved in 1 ml of methanol. As for the test solution, 50 mg of the powdered *P. sidoides* root samples were dissolved in 8 ml methanol.

5.2.2. Metabolomic analysis

The sample preparation, extraction, data acquisition, analysis, data mining and processing were performed by adapting the methods described by Maree and Viljoen (2012), Mediani *et al*. (2012) and Yang *et al*. (2012).

5.2.2.1. Sample preparation and extraction method

The plants were harvested 12 months after planting and the roots were chopped and oven dried (Economy oven, 620 digital, Labotec) at 70 °C for 48 hours. A powdered sample of 50 mg per treatment was weighed in 2 ml Eppendorf tubes for extraction and analysis. Added to the samples was 0.75 mL of CH_3OH-*d4* and 0.75 mL of potassium dihydrogen phosphate (KH_2PO_4), buffered in deuterium water (D_2O) (pH 6.0) containing 0.1% (w/w) TSP (Trimethylsilylpropionic acid sodium salt). The mixture was vortexed at room temperature for 1 min, ultrasonicated for 20 min and then centrifuged for 20 minutes. The supernatant from each tube was transferred to an NMR tube for analysis.

5.2.2.2. Data acquisition/sample analysis

NMR (nuclear magnetic resonance) spectral data were obtained using a 600 MHz ^{1}HNMR spectrometer (Varian Inc, California, USA), with 32 scans recorded. The NMR gives information about the structure of a compound, by placing a substance in a strong magnetic field that affects the spin of its atomic nuclei. A radio wave passes through the substance, reorienting the nuclei; and as the wave is turned off the nuclei releases a pulse of energy or signals that provides data on the molecular structure of the substance (Anand *et al*. 2011). These signals are characterized by their frequency or chemical shift, intensity, fine structure and magnetic relaxation properties, all of which reflects the environment of the detected nuclei (Krishna *et al*., 2005).

The phasing and baseline corrections were conducted using MestReNova software (9.0.1, Mestrelab Research, Spain), with consistent settings for all sample spectra. The chemical shift ranges of δ 4.70 – 4.90 and δ 3.23 – 3.36, representing water and methanol respectively (Mediani *et al*., 2012), were excluded from further analysis.

5.2.2.3. Data mining and processing

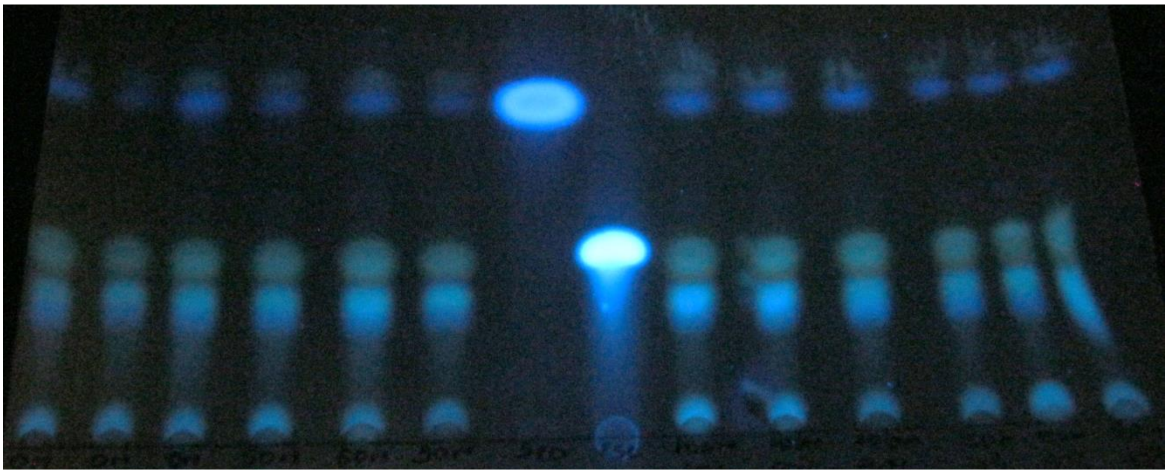
MestReNova software (9.0.1, Mestrelab Research, Spain) was further used for bucketing of NMR spectra. The NMR regions were divided into 0.04 ppm bins resulting in 249 integrated regions.

Thereafter Multivariate data analysis (MVDA) was performed by principal component analysis (PCA), orthogonal partial least square discriminatory analysis (OPLS-DA) and hierarchical cluster analysis (HCA). This was performed with SIMCA-P software (13.0, Umetrics, Sweden) using the Parreto scaling method.

The PCA is an unsupervised analysis performed to provide an overview of all observation on the sample (Maree and Viljoen, 2011). Scatter score plots from the PC analysis were constructed to identify and evaluate groupings, trends and strong outliers (Maree and Viljoen, 2011). The second phase of analysis, the OPLS-DA, is a supervised pattern recognition method of which the main purpose is to separate the systematic variation in the X-matrix into two parts with one part linearly related to the Y-matrix and one that is unrelated to the Y-matrix (Maree and Viljoen, 2011).

5.3. Results

The thin layer chromatography (TLC) results showed the presence of the two standard compounds, scopoletin and esculin, in all the 12 treatments (Figure 5.1). Umckalin was excluded as it is only an indicator compound for *P. sidoides*. The TLC plate however did not seem to indicate any difference in the concentration of the compounds between the samples, as all the treatments showed similar absorbance of UV light. When comparing the standard compounds (1mg/ml) with the compounds in the sample, it is evident that the compounds were found in very low concentrations in the root samples.



N: 0 50 100 150

% ADL: 30 50 70 30 50 70 Scop Esc 30 50 70 30 50 70

Figure 5.1. TLC analysis showing the presences of scopoletin (scop) and esculin (esc) in *P. sidoides* plants exposed to different water and nitrogen treatments. N = nitrogen in kg · ha^{-1}, ADL = allowable depletion level of plant available water.

The results from a principal component analysis (PCA), which is an unsupervised analysis method, did not show good variation between data sets (Figure 5.2). There was no separation between the well watered (30% ADL, green), the moderately stressed (50% ADL, blue) and the severely stressed (70% ADL, red) treatments.

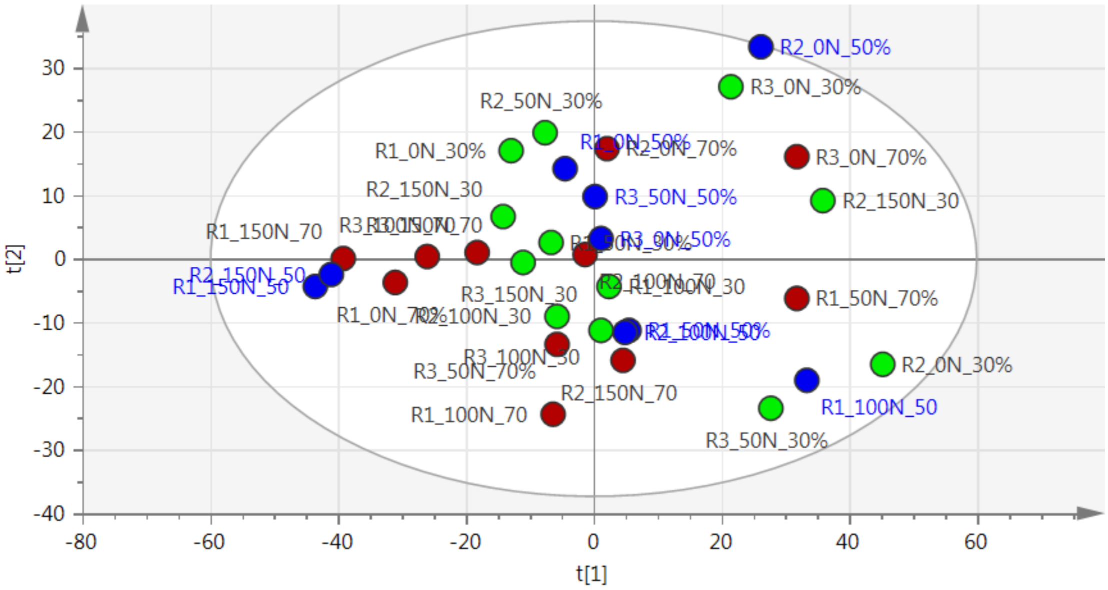


Figure 5.2. Principal component analysis (PCA) results of *P. sidoides* on NMR spectra, with the ellipse representing Hotelling within 95% confidence. The well watered treatments is represented by green colour, moderately stressed by blue colour and severely stressed by red colour.

However, when the data sets were exposed to a supervised method, orthogonal partial least square discriminatory analysis (OPLS-DA), there was a separation observed between the well watered treatment and the water stressed treatments (Figure 5.3). Accordingly the severely stressed treatments are generally clustered to the left of the ellipse, with the moderately stressed treatments more to the centre, indicating a gradual change from the well watered treatments on the right to the severely stressed treatments on the left. Two groups could be observed where the well watered treatment was separated from the two water stressed treatments. The hierarchical cluster analysis (Figure 5.4) further shows similar groupings.

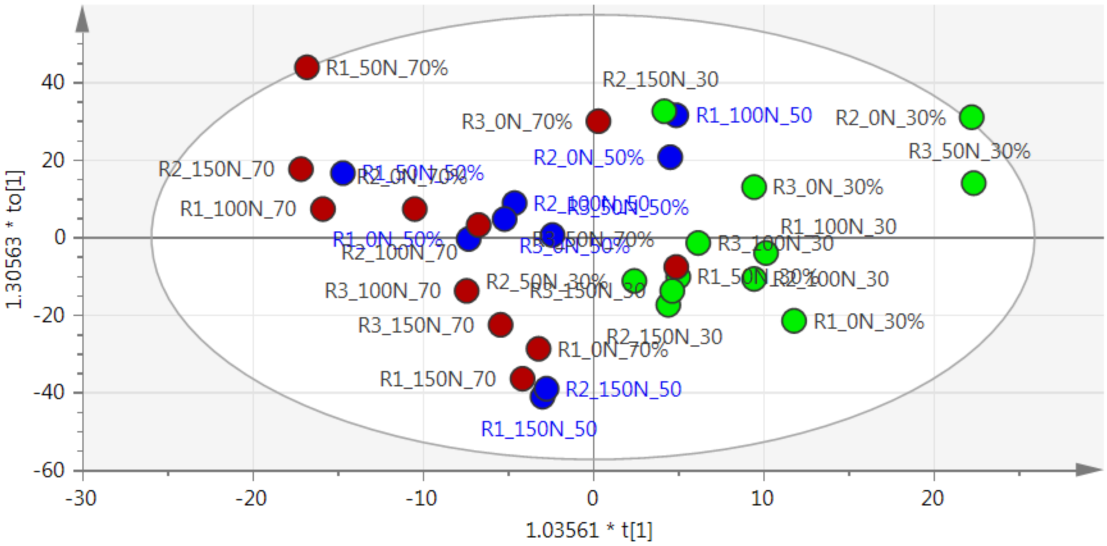


Figure 5.3. Orthogonal partial least square discriminatory analysis (OPLS-DA) results of *P. sidoides* on NMR spectra, with the ellipse representing Hotelling within 95% confidence. The well watered treatments is represented by green colour, moderately stressed by blue colour and severely stressed by red colour.

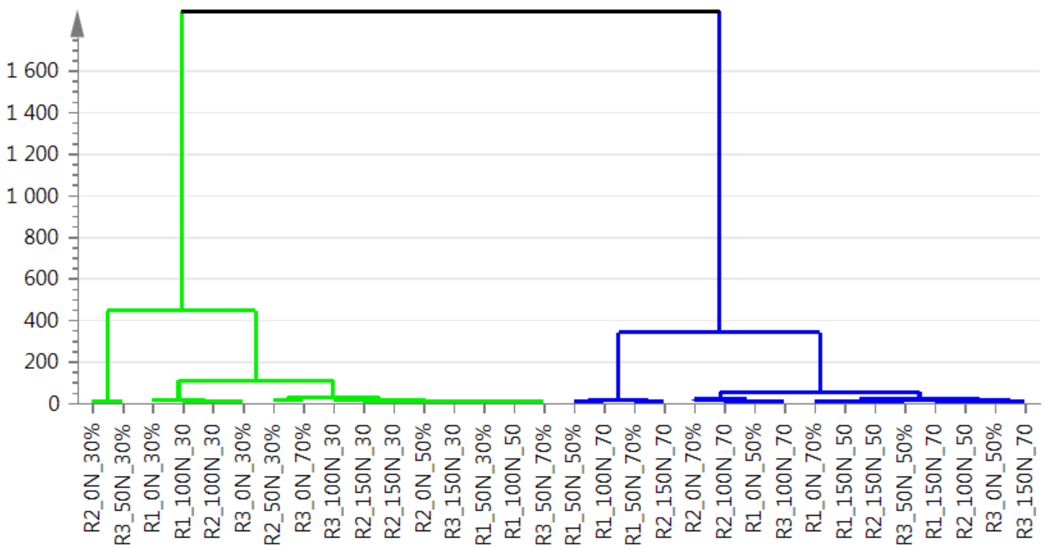
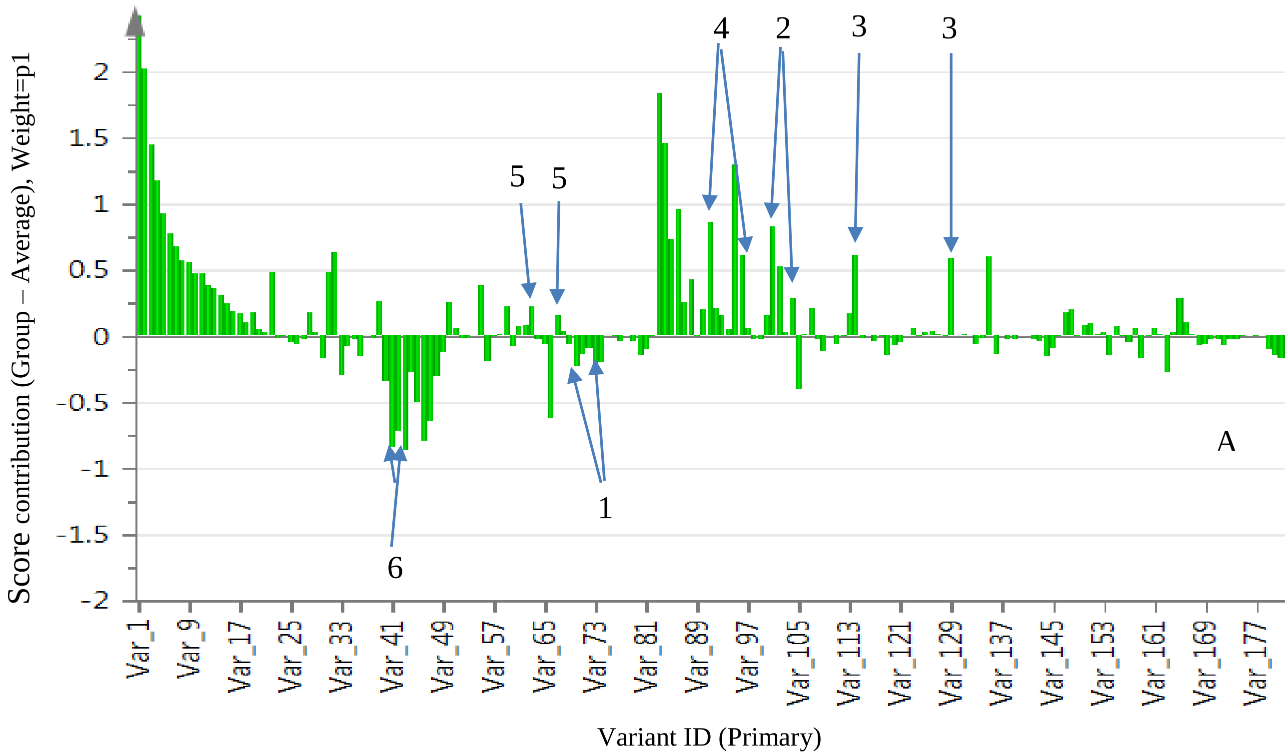


Figure 5.4. Hierarchical clustering showing the two separated groups from results OPLS-DA of *P. sidoides* plants exposed to different water treatments. The green group represents mostly the well watered treatments with none of these well watered treatments in the blue group (water stressed).

Further investigations were performed to reveal the variants which contributed to the separation. Figure 5.5 (A) shows the variants (representing specific NMR regions) which contributed, positively and negatively, to the grouping of the well watered treatment, while Figure 5 (B) shows the variants for the water stressed treatments. The major compounds identified from the contribution plots are asparagine (1), sucrose (2), xylose (3), glucose (4), citric acid (5) and arginine (6), as shown in Table 5.1.



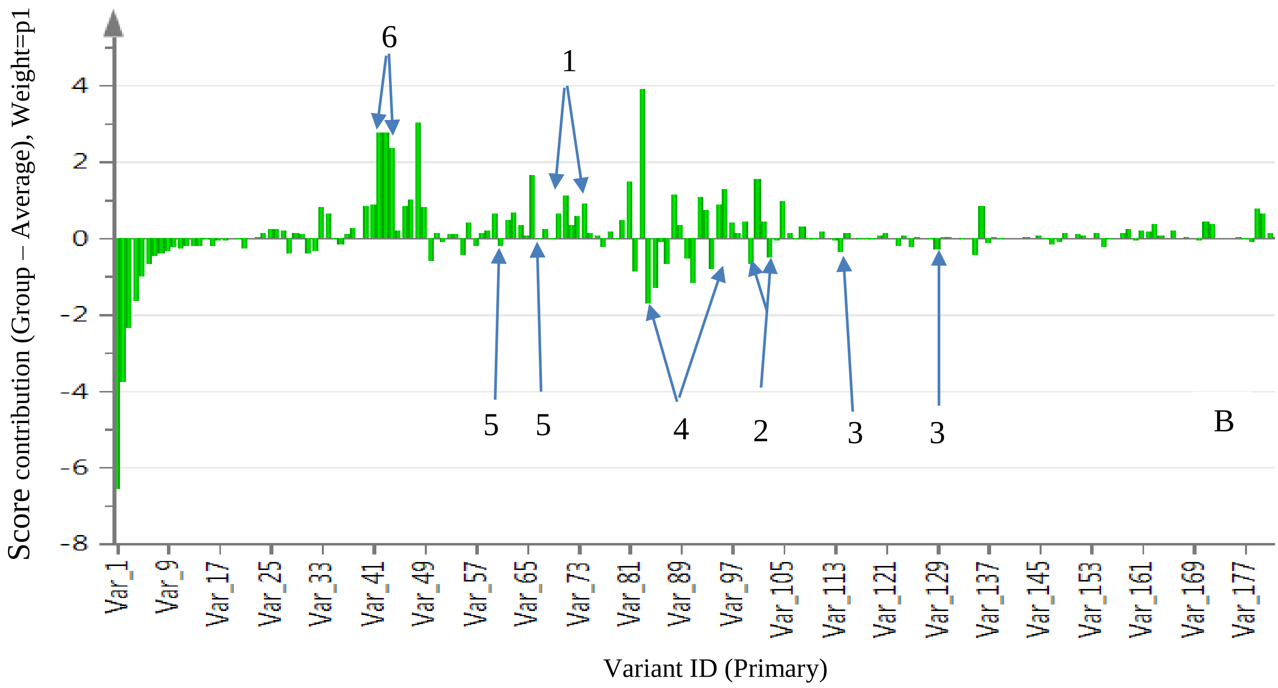


Figure 5.5. Score contribution plot showing NMR regions which had an effect on the separation of the well watered treatments (A) and water stressed treatments (B), in the OPLS-DA. 1 = asparagine, 2 = sucrose, 3= xylose, 4 = glucose, 5 = citric acid, 6 = arginine

Table 5.1. Specific NMR regions and compounds which contributed to the separation of the well watered treatment from the water stressed treatment.

Treatment	Variant	NMR value	Compound	Number
Well watered	32, 34	1.28	Unidentified	-
	54	1.36	Unidentified	-
	56	2.24	Unidentified	-
	57	2.28	Unidentified	-
	63	2.52	Citric acid	5
	67	2.68	Citric acid	5
	85 - 87	3.4 – 3.48	Glucose	4
	89, 90	3.56, 3.6	Glucose	4
	91 - 92	3.64 – 3.68	Glucose	4
	94 – 98	3.76 – 3.92	Glucose	4
	101 – 102	4.04 – 4.08	Sucrose	2
	104 – 105	4.16 – 4.7	Sucrose	2
	115	4.55	Xylose	3
	130	5.15	Xylose	3
	136	5.44	Sucrose	2
	148	5.92	Unidentified	-
	149	5.96	Unidentified	-
	166	6.64	Unidentified	-
Water stress	69 - 74	2.78 – 2.95	Asparagine	1
	99	3.95	Unidentified	-
	41 - 43	1.64 – 1.72	Arginine	6
	46 – 47	1.84 – 1.88	Unidentified	-
	57	2.28	Unidentified	-
	67	2.68	Unidentified	-

Both the PCA (Figure 5.6) and the OPLS-DA analysis (Figure 5.7) on the nitrogen treatments did not show separation between the data sets.

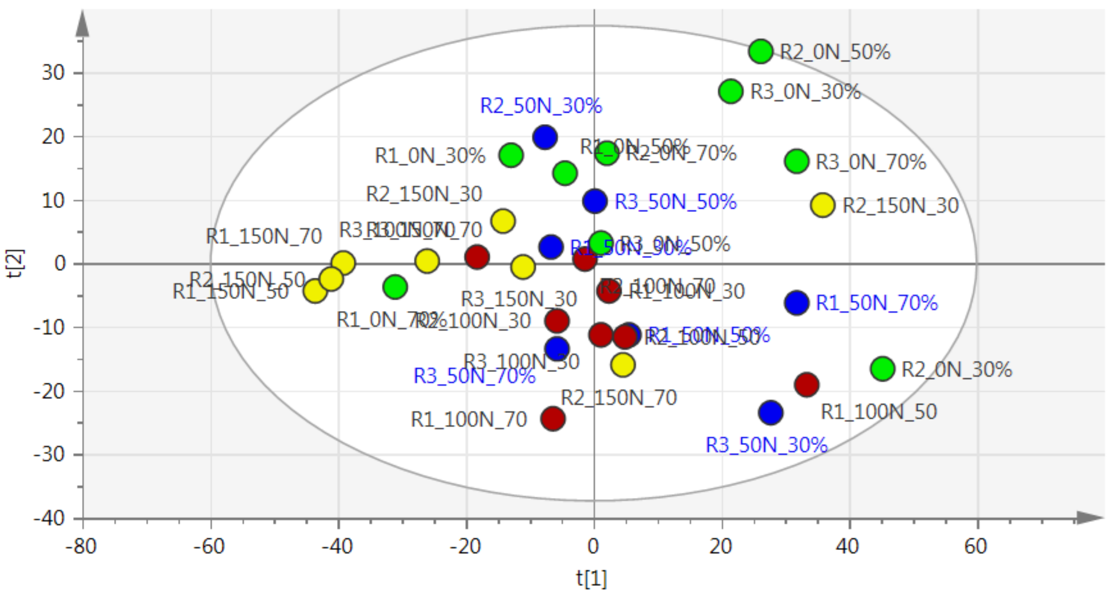


Figure 5.6. Principal component analysis (PCA) results of *P. sidoides* on NMR spectra, with the ellipse representing hotelling within 95% confidence. The N treatments are represented as follows: no nitrogen (green), 50 (blue), 100 (red) and 150 (yellow) kg · N · ha^{-1}.

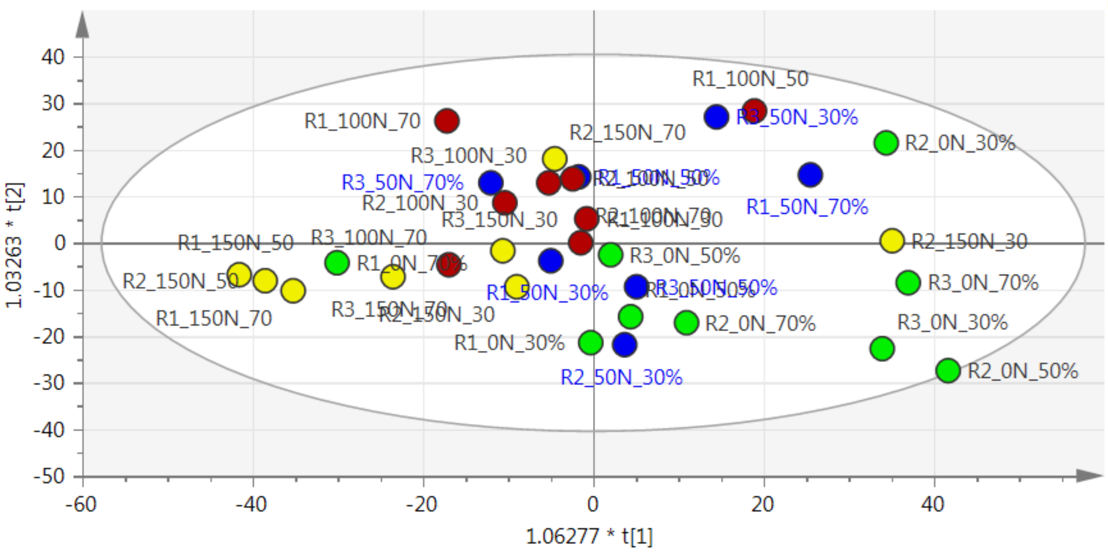


Figure 5.7. Orthogonal partial least square discriminatory analysis (OPLS-DA) results of *P. sidoides* on NMR spectra, with the ellipse representing hotelling within 95% confidence. The Nitrogen treatments are represented as follows: no nitrogen (green), 50 (blue), 100 (red) and 150 (yellow) kg · N · ha^{-1}.

5.4. Discussion

The PCA analysis did not show any separation for water treatments as the data sets were scattered all over in the ellipse. However when the data sets were transformed to the OPLS-DA analysis there were groupings based on largest variation between the samples to separate treatments. Nitrogen did not show any effect on variation on both the PCA and OPLS-DA analysis methods, which is indicative of a very similar metabolite profile with the application of different nitrogen levels.

Although the two main compounds, scopoletin and esculin, were observed for TLC analysis they were not detected for NMR. This could be because both scopoletin and esculin were present at low concentrations which were below the sensitivity threshold (Krishna *et al*., 2005) of the NMR (Shulaev *et al.*, 2008). Yang *et al*. (2012) mentioned that the content of some compounds increases with increasing age of plants, which could explain the low concentrations of the two compounds. In the present study plants were harvested at the age of one year, while harvesters are encouraged to harvest plants that are at least two years old. It seemed however that the two compounds, scopoletin and esculin, did not differ significantly between the treatments and other compounds in higher concentrations were found to be responsible for the separation between the treatments.

The compounds that contributed to the groupings were citric acid, glucose, sucrose and xylose for the well watered treatment, while for the water stressed treatment it was mainly asparagine and arginine. The well watered treatment showed an increase in primary metabolites, especially the sugars. As for the water stressed treatments the amino acids, asparagine and arginine increased.

Stress induces secondary metabolite synthesis in preparation for defense, whereas under no stress primary metabolites will be synthesized, for growth. Accumulation of compounds such as free amino acids, under drought conditions, ensure osmotic adjustment of plants (Simon-Sarkadi *et al.*, 2006 and Lea *et al.*, 2007). Singh *et al*. (1973) reported an increase in concentrations of asparagine, amongst other free amino acids, in the leaves of barley plants exposed to water stress. In potato leaves, no significant differences were observed in sucrose content between the control and water stress treatment, which was induced by application of polyethylene glycol. However, there was a significant increase in the amino acid content in the old leaves of water stressed plants, seven days after inducing the stress (Büssis and Heineke, 1998). This increase was attributed to an increase in asparagine, amongst other amides. Fukutoku and Yamada (1981) also reported a significant increase in amino acid content, especially proline and asparagine, in water stressed soybean plants compared to non-stressed plants. Sucrose and glucose content were also reported to decrease in the leaves and roots of *Lupinus albus* plants exposed to water stress (Pinheiro *et al.*, 2004). Furthermore, asparagine was decreased in the young leaves and roots of *L. albus* due to water stress, though in the stem it was significantly increased (Pinheiro *et al.*, 2004).

5.5. Conclusions

The study successfully differentiated the metabolites responsible for variation between well watered and water stressed root samples of *P. sidoides,* using NMR. This was a quick and easy method applied to obtain a profile of all metabolites present in the samples. The OPLS-DA allowed the separation of the three irrigation treatments into two clusters, the well watered and water stressed (moderately and severely stressed) clusters. However, there was no good separation regarding the nitrogen treatments.

The primary metabolites identified in the well watered treatment were mostly sugars and in the water stressed treatments, amino acids asparagine and arginine. Secondary metabolism, finely tuned, may not be relevant for water stress levels as plants need to survive first. A consideration may be to grow the plants under well watered conditions for a year or two to increase growth and root yield. Thereafter plants can be exposed to water stress in the second season, which is expected to increase the concentration of important secondary metabolites. The lack of information on the compounds responsible for the medicinal activity of *P. sidoides* however poses a challenge in targeting specific compounds in secondary metabolism.

Liquid chromatography-Mass spectrometry (LC-MS) or Gas chromatography-MS (GC-MS) techniques may be more appropriate for identifying and quantifying lower concentration compounds as they are more sensitive than the NMR and should be considered in future studies.

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CHAPTER 6

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

When irrigation must supplement rainfall for crop production, it results in competition between domestic, industrial and agricultural water users. The need for a better understanding of both crop water requirements and its relationship with yield becomes more important in optimization of water application to crops. Thus the determination of exactly when is a shortage or excess of water causing production losses, important for appropriate irrigation management. Irrigation management and nutrient management, especially nitrogen, indirectly affect each other. Under drought conditions root activity is constrained, leading to reduced nitrogen uptake. Nitrogen on the other hand increases vegetative growth, which has an effect on water uptake through an increased transpiration area. The yield and composition of secondary metabolites within a species may vary between plants from different geographical locations and may be influenced by environmental stresses, amongst other factors, and their intensities.

The results from this study showed a relationship between physiological, morphological and yield response as well as chemical composition of *P. sidoides*. The observed stomatal closure under water stress conditions, due to low turgor pressure in the guard cells, had a significant negative effect on leaf area, leaf area index, total biomass and root yield. All the above mentioned parameters decreased with water stress, although there were not always significant differences between moderately stressed and severely stressed treatments. Regulation of stomatal conductance is one of the main mechanisms of reducing water loss under drought conditions, however the uptake of primary substrates for photosynthesis is also compromised. Another response to stress is the reduction in leaf area and leaf number. Any reduction in these further leads to a reduced photosynthetic area. All this resulted in a significant decrease in total biomass and root yield from the well watered treatment to the water stressed treatments.

The chlorophyll content, biomass nitrogen content and nitrogen uptake results showed a significant increase under water stress compared to the well watered treatment. The less turgid cells under water stress could mean that the chloroplasts are closer together, giving a higher chlorophyll content reading on the measured leaf area, while under well watered conditions the open stomata resulted in spaces between the chloroplasts giving a low chlorophyll content reading. It has been reported that under water stress conditions, internal nitrogen concentrations rise due to concentration effect, and this could be because plant growth and development are reduced, thus the build-up of nitrogen in the plant.

The results further showed an increase in the nitrogen use efficiency and while water use efficiency decreased, under well watered conditions. Though the nitrogen uptake was low in well watered treatments, it was efficiently used for growth and development. The significantly increased yields under well watered conditions also resulted in a significantly higher water use efficiency under those conditions.

There was a significant positive effect of nitrogen on number of leaves, but not on leaf area, at the application rate of 100 kg · N · ha^{-1}. The total biomass yield increased significantly with application of nitrogen, compared to the control, with no significance differences between N levels. However, a trend of a decrease in total biomass above 150 kg · N · ha^{-1} was observed and this could indicate a possibility of luxury consumption beyond 150 kg · N · ha^{-1}. The lack of a significant effect of 100 kg · N · ha^{-1}on leaf area and total biomass, which was observed on number of leaves, could possibly mean there was stimulation of a large number of small leaves. Furthermore, nitrogen significantly increased nitrogen content, of the whole plant. This can be linked to the increase in total biomass, with increasing nitrogen level. Nitrogen is known to promote vegetative growth, but it did not translate into an increase in root yield in this study. This could have been due to sufficient residual N in the soil. The nitrogen use efficiency was significantly reduced by nitrogen application beyond 50 kg · N · ha^{-1}and this could possibly emphasize the luxury consumption under increased nitrogen levels.

The investigation into chemical composition of roots showed that it was affected by water stress, but not by nitrogen. Asparagine content, which is an effective molecule for storage and transportation of nitrogen in living organisms, increased with water stress. This could be linked to the increases in nitrogen and chlorophyll contents which was observed under water stress. The increases in total biomass and root yield under well watered conditions could be attributed to the increased primary metabolite content, under such conditions.

Regarding our hypotheses, the following can be concluded:

i. There is an optimum N level (e.g. 100 kg · N · ha^{-1}) that will improve growth and increase yield of storage roots with no negative effect on medicinal active compound levels of the plant.

This hypothesis is <u>rejected</u> as nitrogen level did not have any significant effect on root yield.

ii. Improved root yield will lead to increases in total yield of the medicinally active compounds.

This hypothesis is also <u>rejected</u> as nitrogen application did not have any significant effect on root yield. It is however not yet clear if nitrogen application increased the total yield of medicinally active compounds.

iii. Increasing nitrogen rates will significantly increase chlorophyll content and leaf area.

This hypothesis is also <u>rejected</u> as nitrogen application only had a significant effect on number of leaves.

iv. Appropriate irrigation scheduling will improve yield of storage roots with no negative effect on active compounds, and thus increase the total yield of active compounds.

The hypothesis is <u>accepted</u> as irrigating at 30% ADL of PAW resulted in increased root yield which translate to increased total yield of active compounds.

From this study it can be recommended that further investigations should be conducted, with more sensitive techniques such as Liquid chromatography-Mass spectrometry (LC-MS) or Gas chromatography-MS, to quantify the content of umckalin, scopoletin and esculin. The presence of these compounds has however not been directly linked to the medicinal use of *P. sidoides* although they are generally used as marker compounds. From the NMR metabolomics profile it would seem as if there are also other compounds in high concentrations that could also contribute to the medicinal activity of the plants and therefore more information is needed to conclusively decide if water and nitrogen is affecting the medicinal content and quality of the plant material. What is important to note is that the major differences observed between the treatments are due to primary metabolites present and that the role of these compounds might be overlooked in the search for secondary metabolites, which might be present in very low concentrations. It is evident that the plant must ensure survival first after a change in environmental conditions, which can be seen in the major change in primary metabolite content of well watered treatment compared to the water stressed treatments. The increase in primary metabolites also shows that the plant is growing better than in the water stressed conditions. The general perception that secondary metabolism is affected by environmental conditions is not supported by the evaluation of the two marker compounds in the study. The compounds are generally accepted to be present in the root samples of *P. sidoides* and *P. reniforme*, but the actual contribution to the medicinal activity and therefore quality of the extract has not been confirmed yet. The information can then be used to confirm if the reduced root yield under water stress is not compensated for by an increase in content of the mentioned active compounds and only then can recommendations be made to farmers. Further research under different soil conditions and other nutrient stress factors should also be investigated. A further recommendation is that plants should be grown for two years or more before harvesting to ensure higher levels of secondary metabolites when stressed.

Appendix 1

Randomisation of the trial, with the blocks representing replicates. N = nitrogen in kg · ha^{-1} and allowable depletion level (ADL) as percentage (%) of plant available water.

File name is Pelargonium sidoides randomisation.gen

To determine the effect of different N levels and soil water depletion regimes
on yield and chemical composition of P. sidoides
Randomised block design
3 sets (blocks or reps)
========== of 12 random numbers for 4 N x 3 ADL

TMTCOMB	N	ADL
1	0	30
2	0	50
3	0	70
4	50	30
5	50	50
6	50	70
7	100	30
8	100	50
9	100	70
10	150	30
11	150	50
12	150	70

===== BLOCK 1 =====

POSITION	TMTCOMB
1	7
2	4
3	1
4	10
5	3
6	11
7	12
8	6
9	2
10	5
11	8
12	9

===== BLOCK 2 =====

POSITION	TMTCOMB
1	4
2	6
3	10
4	12
5	3
6	8
7	5
8	9
9	1
10	2
11	11
12	7

===== BLOCK 3 =====

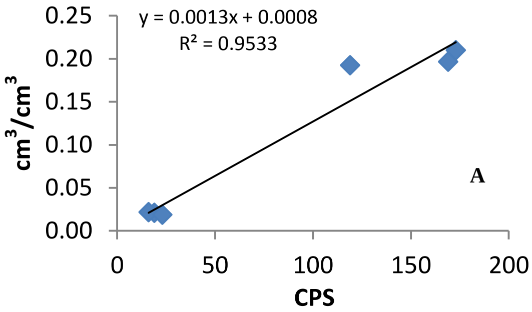
POSITION	TMTCOMB
1	6
2	10
3	3
4	2
5	12
6	11
7	5
8	1
9	9
10	4
11	7
12	8

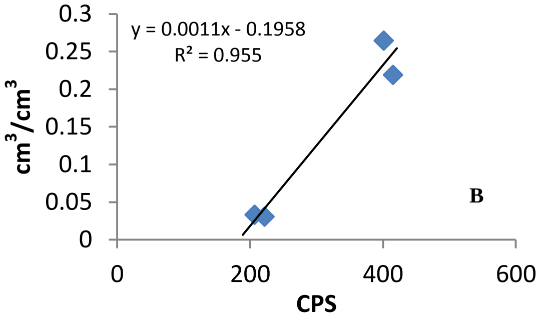
End of Meshack Mofokeng - ARC-VOPI - Project no a070202b. Current data space: 1 block, peak usage 1% at line 33.

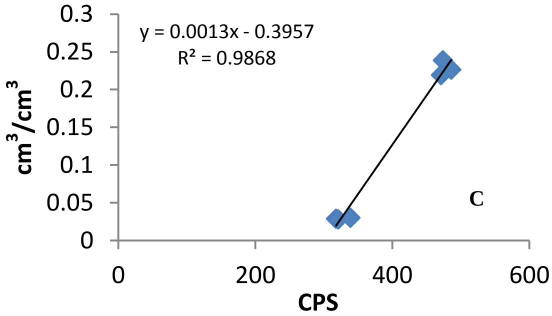
GenStat 64-bit Release 14.1 (PC/Windows 7) 16 April 2012 08:56:58
Copyright 2011, VSN International Ltd.
Registered to: ARC Biometry Unit

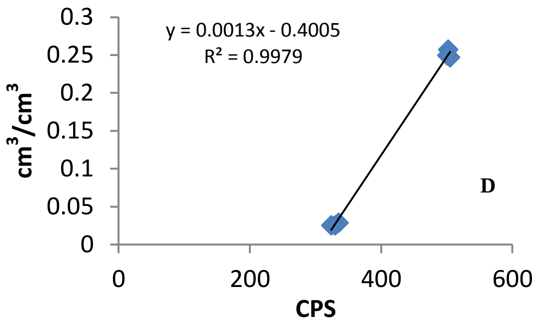
Appendix 2

The calibration curves that were used to determine the soil water loss at 0 - 0.2 (A), 0.2 - 0.4 (B), 0.4 - 0.6 (C), 0.6 - 0.8 (D) and 0.8 - 1.0 m (E) of the soil profile.








y = 0.0013x - 0.4005
R² = 0.9979
cm³/cm³
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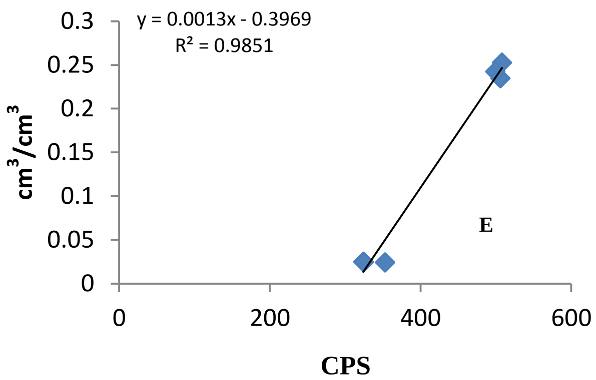
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
CPS
D


y = 0.0013x - 0.3969
R² = 0.9851
cm³/cm³
0.3
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
CPS
E

Appendix 3

Plant identification dispatch list: SANBI (South African National Biodiversity Institute).

SANBI

Biodiversity for Life

Pretoria National Herbarium (PRE)

South African National Biodiversity Institute

Private Bag X101, Pretoria, 0001

South Africa

Tel: +27 12 8435000, Fax: +27 12 8043211

140^{02} Plant Identification Dispatch List 17 March 2013

Client	Meshack Mofokeng	
Address	P/BAG X 293	Tel: 012-841-9611
	PRETORIA	Cell:
		Email: mofokengm@arc.agric.za

ID CODES:

1 = Specimen too poor to ID
5 = Genus requiring/under revision
2 = Label information inadequate
6 = Specimen closest to name listed (cf)
3 = Cannot match specimen in herbarium
7 = Please send more material
4 = Specialist not available to do ID
8 = Please refer to attached note/letter
9 = New record

Collector	No.	Plant Name	Det. By	Det. Notes	ID Code
M, Mofokeng	P.fi.	*Pelargonium sidoides* /.DC'	Masupa. T.T. I7/3/2013		0

Please note a handling fee is charged for each specimen received for identification 1

Appendix 4

Preparation of treatment plots under rainshelter (4.a and b), the trial after planting (4.c) and *P. sidoides* plants under well watered (4.d), moderately stressed (4.e) and severely stress conditions (4.f).