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Environmental stress effects on the phytochemistry and bioactivity responses of a South African medicinal bulbous plant, *Tulbaghia violacea* Harvey (Alliaceae)

by

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DECLARATION

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ABSTRACT

Deteriorating living and environmental conditions have contributed to the increasing prevalence of diseases in plants and animals. In humans, accumulation of abnormally high levels of free radicals in the tissues has been implicated in many non-communicable diseases, such as diabetes, cancer, arthritis, ischemia, gastritis, obesity and asthma. Worldwide, there is recognition of need to improve plant and animal health. *Tulbaghia violacea* (Alliaceae) is a medicinal plant that is extensively harvested by traditional healers in the wild for its medicinal uses and if this practice continues, it may result in an unsolicited decline of the species in situ. Therefore, there is a need for cultivation of this species. Plant cultivation in a controlled environment for conservation purposes as well as the enhancement of yield and quality is gaining favour among farmers and consumers. The main aim of this study was to investigate the effects of altering the growing conditions by applying environmental stresses on the plant growth, antifungal and antioxidant activities of *T. violacea,* with the view of enhancing the future cultivation of this species for pharmaceutical companies, traditional healers and the horticulture industry.

This study was divided into two parts, and the first part, which was further sub-divided into two separate preliminary experiments, is presented in chapter three. Simultaneous assessments of the effects of i) varied pH levels (pH 4, pH 6, pH 8) and ii) light intensity on plant growth, antioxidant-content and -capacity of extracts of *T. violacea* were carried out. The second part of the thesis consisted of a more detailed assessment of the above-mentioned independent variables and interactions thereof on plant growth, and antifungal activity of extracts of *T. violacea.* Results obtained from the first part of the study, showed that plants exposed to pH 6 showed a marked increase in plant height (from 25-37 cm) after 2 months of treatment although, generally, the variations of the different growth parameters among the pH treatments were not significant ($p > 0.05$). Antioxidant-contents and -capacity were not significantly different ($p > 0.05$) when pH treatments were compared. However, a high polyphenol content value (of 3 mg/g) occurred in leaves of plants exposed to pH 8. Overall, comparatively, there was no significant difference ($p > 0.05$) in antioxidant-content and -capacity when pH treatments. In the light experiment, decreasing light intensity led to the elongation of plant height. A higher mean shoot length of 34.6 cm was obtained under low light compared to normal light (26.5 cm) two months post-treatment. The results obtained in this study indicated that light had a significant affect ($p < 0.05$)

on the vegetative growth of this species. In contrast, normal light intensity yielded higher antioxidant-content and -capacity. The polyphenol and flavanol content were fluctuating between the averages of 5.8 mg/g to 8.5 mg/g. Overall, there was a significant difference ($p < 0.05$) in the antioxidant-content and -capacity when low and normal light intensity treatments compared. In conclusion, both normal light intensity and at pH 8 induced better antioxidant results. In the second part of the study, chapter four, one-month old *T. violacea* plantlets were grown under two light intensities (low light and normal light) in a greenhouse and concurrently exposed to varying pH levels: pH 4, pH 6 and pH 8. Plants exposed to normal light received natural sunlight through the roof of the greenhouse, while low light intensity (40% reduction) was achieved using shade nets. Plants were drip irrigated with Nutrifeed fertilizer. Plant growth parameters such as height and fresh and dry weights were determined. Leaf samples were analysed for macro-and micro-nutrients contents. Antifungal tests were carried out on the plant extracts from the various treatments in an antifungal bioassay (minimum inhibitory concentration [MIC]). The experimental data collected were analysed using one and two-way analyses of variance (ANOVA), and Tukey HSD was used to separate the means at $p < 0.05$ level of significance. Varied effects of different pH levels (4, 6 and 8) and light intensities (low and normal) on plant height, and fresh and dry weights were recorded in the current study. A significant interactive (df, 2; $F = 0.001$; $p < 0.001$) effect between pH and light on fresh weight was observed. The results revealed that there was a significant difference (df, 2, 57; $F = 12.63$; $p < 0.001$) in dry weights with plants under normal light intensity and pH 4 treatment (8.285 ± 0.802 g) producing the highest dry weight. There was a significant interaction (df, 2; $F = 6.4$; $p < 0.001$) between pH and light intensity on plant dry weight. Extracts from plants grown under normal light intensity showed stronger antifungal activity at pH level 4, and MIC values ranged from 0.18 ± 0 to 0.375 ± 0.04 mg/ml at 6h and 1.5 ± 0 to 0.97 ± 0.18 mg/ml at 18h. In conclusion, this study demonstrated the interactive effects of pH and light intensity on the growth of *T. violacea*. These findings also confirmed that it is possible to enhance the cultivation of *T. violacea* under greenhouse conditions. Chapter 5 focused on the interactive effects of pH and watering regime on plant growth, nutrient uptake and antifungal activity of *T. violacea* plant extracts, grown hydroponically. The results showed that there were significant differences ($p < 0.05$) on plant growth parameters amongst the different watering regimes under normal light intensity. Broadly, two trends occurred in the results: firstly, more macro-nutrients were taken up by plants in the

higher frequency watering intervals as opposed to higher tissue micronutrient nutrient values for plants grown under the lower light intensity conditions. The levels of N, P, K, Mg nutrient uptake differed significantly in plants ($p < 0.001$) among watering interval periods. On the other hand, plants simultaneously exposed to extended watering intervals of 21-day and low light intensity showed more bioactivity of the crude extracts against *F. oxysporum* in the MIC bioassay. Based on the current results, a combination of shorter watering interval and normal light intensity favoured plant growth and development, while plants grown under low light intensity with longer watering interval showed good bioactivity.

Broadly, these results demonstrated that varying pH, light intensity, and watering regime can influence plant growth, secondary metabolite contents and antifungal activity of crude extracts of *T. violacea*. These findings will contribute to the current body of knowledge around cultivation of indigenous medicinal plants. The study will further benefit the conservation of medicinal plant initiatives, increased income of small-scale farmers and potentially promote indigenous knowledge by increasing the availability of South African medicinal plants.

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DEDICATION

This work is dedicated to:

- My late Grandfather Goodman Bhuti Ncise, I know if you were still around you would have been proud of me.
- To my Mother Nwabisa Portia Ncise, for her precious support, love, patience and encouragement.

Contents

List of acronyms

°C	Degree Celsius
ABTS	2,2 -azino-di-3-ethylbenzthiazoline sulphonate (ABTS)
ANOVA	Analysis of Variance
B	Boron
Ca	Calcium
Cu	Copper
Fe	Iron
FRAP	Ferric reducing antioxidant power
HCl	Hydrogen chloride
K	Potassium
LECA clay	Light Expanded Clay Aggregate
MIC	Minimum Inhibitory Concentration
Mg	Magnesium
Mn	Manganese
N	Nitrogen
ORAC	Oxygen Radical Absorbance Capacity
Na	Sodium
RH	Relative Humidity
S	Sulphur
TA	Total Activity

P	Phosphorus
pH	Potential hydrogen
PPFD	Photosynthetic photon flux density
Zn	Zinc

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Mankind has relied on plants for centuries as primary sources for food and to provide for their medicinal needs (Van Wyk et al., 2009). Humans still use plants for culinary, nutritive, aromatic and medicinal purposes. The informal and commercial sectors cultivate medicinal plants to generate income (Van Wyk et al., 2009). In South Africa, it is estimated that about 200 000 indigenous traditional healers utilize indigenous plants for traditional medicine, and approximate 60% of South Africans consult these healers for their healthcare needs. Approximately 4000-10000 wild populations of medicinal plants are endangered due to over-harvesting (Van Wyk et al., 2009).

The rapid population growth in developing countries has led to habitat degradation, loss of genetic diversity and local species extinction (Lefever et al, 2013). The growing problem in Africa is the over-exploitation of plants species for medicinal purposes since medicinal plants are still collected from wild populations. In contrast, in Europe, China and India medicinal plants are often cultivated on a large scale to meet the growing demand for herbal medicine (Zschocke et al., 2000). Even though the protection of these species may be achieved through an increase in regulations and sustainable methods of harvesting, the most important long-term goal is to increase alternative methods of cultivation (Fennel et al., 2004).

There are various cultivation methods used by biotechnologists and horticulturists to optimize yield, achieve uniformity and high quality products (Goins et al., 1997). These alternative methods include tissue culture, hydroponic and special breeding programs. Medicinal plants can be cultivated on a commercial scale by the use of hydroponic systems in a controlled environment (Matanzima, 2016). With hydroponic, the secondary metabolite constituents can be easily manipulated because of the controlled growing environment. Furthermore, it is one of the strategies used for conservation of endangered species (Hayden, 2006). Studies have shown that the manipulation of environmental conditions and nutrient concentrations can lead to increased secondary metabolite production (Matanzima, 2016). In hydroponic, the favourable conditions for plant cultivation can be easily controlled and monitored, and this

may improve the quantity and quality of secondary metabolites that are essential for the neutralization of excess free radicals in humans (Mossi et al., 2011).

The first objective of this study was to carry out a preliminary evaluation of the effects of varying pH, light and watering regimes on the growth and antioxidant activity of *Tulbaghia violacea* (Alliaceae), cultivated in hydroponicunder greenhouse conditions. The second objective was to assess the interactive effects of light and pH on plant growth, nutrient uptake and antifungal extracts of *T. violacea* plants grown in hydroponic. The third objective was to assess the interactive effects of pH and watering regime on plant growth, nutrient uptake and antifungal of extracts of *T. violacea* plants grown in hydroponic. The results of this study could benefit pharmaceutical companies, traditional healers and the horticulture industry in the enhancement of the medicinal properties of this species. In addition, the hydroponic cultivation approaches that were applied in this study could optimize the survival of this species.

1.2 Statement of the research problem

Deteriorating living and environmental conditions have led to an increase in the prevalence of diseases in plants and animals. In humans, accumulation of abnormally high levels of free radicals in the tissues has been implicated in many non-communicable diseases in humans, such as diabetes, cancer, arthritis, ischemia, gastritis, obesity and asthma (Cook and Samman, 1996; Kumpulainen and Salonen, 1999). Free radicals also influence susceptibility of humans to many communicable diseases, including AIDS and tuberculosis (Daniels et al., 2011). For plants, the adoption of monocultures and the excessive use of synthetic antimicrobial agents in agriculture have resulted in increased parasitism of plants by microbes. Worldwide, there is recognition of the need to improve plant and animal health. Cultivation systems that ensures efficient nutrient uptake by plants could lead to rather optimal quantities of macronutrients and micronutrients in plant tissues, resulting to improvement of crop production.

Medicinal plants have been used for many centuries by man for treatment of human and livestock diseases (Van Wyk et al., 2009). The demand for medicinal plants continues to be high for many reasons: medicinal plants are an important source of bioactive agents in modern medicine used in traditional medicine, affordable and considered by many as less toxic (Katerere & Eloff, 2008). Medicinal plants will always be needed by humans to combat

diseases of plants and animals. Because of its healing properties, *T. violacea* is one of the most utilized plants by traditional healers in KwaZulu-Natal. It has shown promising antimicrobial and antioxidant results when tested against some bacterial, nematodes and fungal diseases (Malungane, 2014). It is difficult to find *T. violacea* outside protected areas because it is highly utilized by traditional healers (Aremu and Van Staden, 2013). The high demand for medicinal plants has favoured the over-exploitation and harvesting in the wild, thus, requiring the cultivation of these species to alleviate the risk of extinction (Fennel et al., 2004). The commercial production of this plant can be done in a controlled environment, such as a greenhouse. There is inadequate information pertaining to cultivation requirements of this species (Van Den Heever et al., 2008). In this study, the aim was to determine the effects of different environmental variables (independent), such as shading, watering regimes and pH on plant growth, in a controlled environment, using hydroponics. Furthermore, the effect of these environmental variables were also measured on antioxidant and antifungal activity, with the view of optimizing the medicinal properties of *T. violacea*. Another important extrinsic factor is potential hydrogen (pH). It plays a major role on plant growth in the soil by influencing the availability of various nutrients for plant uptake (Peterson, 1983; Kunh et al., 1995; Marschner, 1995). The mechanisms through which external stresses influence plant production of secondary metabolite are being unmasked. For example, subjecting plants to low light and high nutrients could result in low carbon to nutrient ratio and consequently, decreased concentration of secondary metabolite since plants will allocate most of their photosynthetic by-products for growth processes (Bryant et al., 1983; Cronin and Hay, 1996).

1.3 Hypotheses

I. Varying pH, light and watering regime will influence plant growth and the antioxidant-content and -capacity of *T. violacea* under greenhouse conditions.
II. Light and pH will have interactive influences on plant growth and antifungal properties of *T. violacea* under greenhouse conditions, i.e. in reducing the growth of the fungus, *Fusarium oxysporum*.
III. Varying light intensity and watering regimes will have interactive influences on plant growth and antifungal properties of *T. violacea* under greenhouse conditions.

1.4 Objectives of the research

The main aim of this study was to investigate the effects of altering the growing conditions by applying environmental stresses on the plant growth, antifungal and antioxidant activities of *T. violacea,* with the view of enhancing future cultivation of this species for pharmaceutical companies, traditional healers, and the horticulture industry.

1.5 The specific objectives

To carry out a preliminary evaluation of the effects of varying pH, light and watering regime on growth and antioxidant content and capacity of *T. violacea* cultivated hydroponic under greenhouse conditions

To assess the interactive effects of light and pH on plant growth, nutrient uptake and antifungal of extracts of *T. violacea* grown hydroponically.

To assess the interactive effects of pH and varying watering regime on plant growth, nutrient uptake and antifungal of extracts of *T. violacea* grown hydroponically.

CHAPTER TWO
BACKGROUND AND LITERATURE REVIEW

2.1 Introduction

Tulbaghia violacea Harvey (Alliaceae) is a medicinal plant that is regularly harvested by traditional healers in the wild and if this practice continues, it may result in a serious decline of the species (Zschocke et al., 2000; Jager and Van Staden, 2005; Mander and Mckenzie, 2005; Naidoo et al., 2008; Van Wyk et al., 2009). Although it is still listed as 'Least Concern' on the 'National Red list of South Africa', it may eventually become threatened with extinction because of its high demand (Mander, 1999; Raimondo et al., 2009). Despite its high medicinal value, only a few studies have focused on *T. violacea*. Ncube et al. (2013) evaluated antibacterial and antifungal properties of extracts from micro-propagated and outdoor grown *T. violacea,* and found that micro-propagated plants produced increased levels of antibacterial activity compared to outdoor grown plants. In this study, *T. violacea* plants was pH-stressed during hydroponic cultivation in an attempt to enhance its antifungal properties and these extracts were tested against the fungus, *Fusarium oxysporum*. Pathogenic strains of this fungus have been isolated from HIV/AIDS patients; hence, results from this research could possibly contribute to current HIV/AIDS research programs (Ncube et al., 2011).

Factors such as land availability, water availability, season, climate, pests, and diseases are major concerns during the conventional cultivation for conservation purposes of indigenous plant species (Pierik, 1987; Arikat et al., 2004). However, modern greenhouse and hydroponic technologies can be constructively used for conservation of threatened plant species and to manipulate the production of phytochemicals in medicinal plants. Phytochemicals or secondary metabolites are chemicals that are produced by plants for their protection against insect attack, plant diseases, environmental stress, and many other purposes. These phytochemicals can be used as remedies against certain human diseases. Hydroponics is one of the biotechnoloical cultivation methods that are commonly used for the manipulation of secondary metabolites (Gontier et al., 2002). It was applied in this study in an attempt to enhance the production of secondary metabolites as well as to ensure further conservation of the species.

2.2 The genus *Tulbaghia* and its distribution

The family Alliaceae consists of 30 genera and about 600 species (Adayemi et al., 2013), and about 63 known *Tulbaghia* species have been identified by the Kew World Checklist of Selected Plant Families (KWCSPF) and the World Checklist of Selected Plants (WCSP) (2013). The family of Alliaceae is closely related to Liliaceae and Amaryllidaceae based on their taxonomy (Aremu &Van Staden, 2013). The genus is widely distributed in Asia, Mediterranean Europe, North and South America, and southern Africa. Historically, the genus *Tulbaghia* was named after the death of Ryk Tulbagh (1699-1771), a Dutch governor of the Cape of Good Hope (Watt and Breyer-Brandwijk, 1962; Pooley, 1998). For centuries, *Tulbaghia* spp. were used for their medicinal, nutrition and ornamental values in Africa (Aremu & Van Staden, 2013). The genus remains one of the most economical species with medicinal, nutrition and ornamental potentials (Reinten et al., 2011; Van Wyk, 2011a; Van Wyk, 2011b). South Africa has about twenty species that belongs to the *Tulbaghia* genus, which are morphologically closely related to the Allium genus, which is also a member of the Alliaceae family (Jacobsen et al., 1967). *Tulbaghia capensis, T. cominsii, T. violacea, T. dregeana, T. galpinii, T. simmleri* and *T. acutiloba* are species found on the rocky grassland in KwaZulu- Natal, Gauteng, Eastern Cape and Western Cape region (Van Wyk et al., 1997; Van Wyk and Gericke, 2000; Nguyen, 2008). *T. violacea* naturally occurs in the Eastern Cape, Western Cape and parts of Kwazulu-Natal (Van Wyk et al., 1997). In Africa, the natural distribution occurs in Botswana, Tanzania, Malawi and southern African countries (Lyantagaye, 2011). Generally, the genus can grow in a range of areas from rocky to semi-desert to boggy areas (Aremu & Van Staden, 2013).

2.3 Morphology and propagation

Tulbaghia violacea is commonly known as wild garlic, society garlic, sweet garlic, wilde knoffel (Afrikaans) and itswele lomlambo (Xhosa) (Kubec et al., 2002; Harris, 2004). *Tulbaghia violacea* is an evergreen, fast growing, bulbous, perennial plant that can reach 0.5 m in height. It can grow easily in most soils and can tolerate prolonged drought, although the plant will flourish with regular watering (Harris, 2004).

The fragrance of *T. violacea* resembles that of *Allium sativum* (garlic) when leaves are crushed due to the presence of cysteine-derived sulphur compounds, which are found in both species (Van Wyk et al., 2009; Jacobsen et al., 1968). Hence, due to the garlic fragrance, it

can also be used for food flavouring (Van Wyk et al., 1997; Kubec et al., 2002; Harris, 2004). It produces a tall flower stalk from January to April with tubular, pinkish, mauve flowers that are clustered into umbels of up to twenty flowers (Harris, 2004). The flat, hard, black seeds are produced in triangular fruits that are grouped into a head (Van Wyk et al., 1997). *Tulbaghia violacea* is propagated either by seed or division of the large clumps of bulbs, which can then be planted individually (Van den Heever, 2006).

Figure 2.1 Photography of *T. violacea* showing pink inflorescence and morphology (Source: http://www.plantbook.co.za/tulbaghia-violacea/)

2.4 Medicinal uses

In South Africa, *T. violacea* bulbs and leaves are traditionally utilised for treatment of gastrointestinal ailments, asthma, fever, tuberculosis and the leaves are used to treat cancer of the oesophagus (Kulkarni et al., 2005; Van Wyk et al., 2009). However, studies show that side effects such as gastroenteritis, abdominal pain and inflammation may result due to excessive consumption of the plant (Van Wyk et al., 1997; Van Wyk and Gericke, 2000).

Research done by Raji (2012) indicates that *T. violacea* can lower blood pressure and the heart rate. South Africa is currently facing elevated rates of HIV/AIDs cases and investigations continue on medicinal plants for the screening of anti-HIV agents (World Health Organisation, 1989; Motsei et al., 2003; Klos et al., 2009). *Tulbaghia violacea* has shown promising antimicrobial activity against some medically important pathogenic bacteria and fungi that cause infections in HIV/AIDS patients (McGaw et al., 2000; Gaidamashvili & Van Staden, 2002; Motsei et al., 2003; Klos et al., 2009). The Zulu people of South Africa

use it to repel snakes in their gardens and houses (Van Wyk et al., 1997; Maoela, 2005). It can also be used as a treatment for infant and fantanelle disease (an anatomical feature of the infant human skull comprising any of the soft membranous gaps (sutures) between the cranial bones that make up the calvaria of a fetus or an infant) (Maoela, 2005). The Rastafarians eat a mixture of this plant and chillies to stop aches and pains and to keep them warm during winter seasons (Maoela, 2005). Traditional healers boil fresh bulbs and leaves in water and soak it for a day to treat fever, arthritis and rheumatism (Van Wyk et al., 1997; Maoela, 2005).

2.4.1 Antioxidant activity

Plant extracts are regularly used as rejuvenators, tonics and nutritional supplements to promote a healthy lifestyle (Aremu and Van Staden, 2013). Antioxidants found in plant extracts can prevent free radicals from damaging tissue or organs in the body (Adewusi et al., 2011). Pietta (1998) defines oxidation as the transfer of electrons from one atom to another and represents an essential part of aerobic life and our metabolism. The phenolic compounds associated with antioxidant activity play a significant role in adsorbing and neutralizing free radicals (Zheng and Wang, 2001). Free radicals are atoms that contain more than one unpaired electron, and the unpaired electrons can react with oxygen to form superoxide that might be harmful to human tissue. Unhealthy diets as consumed by humans such as spicy junk food, cigarette smoking and exposure to atmospheric pollutants can create more free radicals in the body. When the body is exposed to high levels of free radicals, the antioxidants decreases. Hence, free radicals are associated with pathophysiological ailments such as atherosclerosis, arthritis, and ischemia, reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDs (Cook and Samman, 1996; Kumpulainen and Salonen, 1999).

The diet of fruits and vegetables humans consume can help to neutralise free radicals in the body. However, there is still a wide interest in potential medicinal plants that can reduce the effects of free radicals on tissue injury in the body (Schuler, 1990). Furthermore, crude extracts of *T. violacea* have shown great medicinal potential against the nematode *Caenothabditism elegans* at concentration of $2mg/ml^{-1}$ after 7 h (McGaw et al., 2000).

The leaves of *T. violacea* (specimens found in the Transkei and Kwa-Zulu Natal areas) are consumed as a substitute for spinach and are a rich source of micronutrients (Aremu and Van

Staden, 2013). Micronutrients such as Vitamin C, Vitamin E, Boron, and Beta-carotene in diets contribute to the scavenging and reduction of free radicals (Lin et al., 1999; Opoku et al., 2000). This proves that *T. violacea*'s antioxidant activity can prevent, slow or minimize the damage caused by the free radicals. This study was focus on possibly enhancing the antioxidant-content and -capacity following exposure of plants to varying pH levels and low or high light intensity of *T. violacea*.

2.4.2 Antifungal activity

In South Africa, *T. violacea* bulbs and leaves are traditionally utilized for the treatment of gastrointestinal ailments, asthma, fever, tuberculosis and the leaves are used to treat cancer of the esophagus (Kulkarni et al., 2005; Van Wyk et al., 2009). *T. violacea* is one of the most harvested plants in the wild for its medicinal properties. Traditional healers mostly use freshly prepared *T. violacea* for a decoction against oral fungal infections. There is a need to cultivate this species in controlled environmental conditions in order to conserve and facilitate rapid production of antifungal properties regardless of the seasonal and climate changes. In a previous study done by Ncube et al. (2011) by comparing micropropagated and outdoor cultivated *T. violacea*, it was demonstrated that micropropagated plants had great antibacterial activity against *Bacillus subtilis*. *T. violacea* has similar antibacterial and antifungal activities as commercial garlic (Martindale, 1993; Brunetonn, 1995). *Allium sativum*'s main pharmacological properties are bactericidal, virucidal, fungicidal and anti-parisitic. The compounds found in commercial garlic are linked to the presence of organic sulphur compounds, including thiosulfates. Therefore, the objective of this study was to adapt the watering regime, light conditions and nutrient solution pH to determine its effects on the antifungal activity of *T. violacea*.

2.5 Cultivation of medicinal plants

2.5.1 Hydroponics

The Greek words '*hydro*' means water and 'ponos' means labour, which forms the basis of the word 'hydroponics' (Jones et al., 1998; Resh, 1998). The hydroponic system is a soilless cultivation method where plant roots absorb nutrients from a water solution for their growth

(Bugbee, 2003). In the hydroponic system, water can be re-used (conservation) and land is protected from contamination (Murali et al., 2011). By using the hydroponic system, plants that are out of season can be grown, and a high-density maximum crop yield can be achieved in a short period of time (Jehnson, 1999; Koohakan et al., 2004). Hydroponics can also be used to increase uniformity, increase plant vigour and decrease the levels of infection by plant pathogens (Canter et al., 2005). The Hanging Gardens of Babylon and the Floating Gardens of the Aztecs in Mexico were using the hydroponic cultivation technique centuries ago (Jones, 1998). In World War 2, large amounts of fresh vegetables that were supplied to troops stationed in and around the islands in the western pacific were produced by means of hydroponics (Jones, 1998). In recent times, hydroponics has become a viable alternative method for growing plants to preserve water using different techniques such as the drip system, Nutrient film technique , sub-irrigation and others (Lefever, 2013).

2.6 Influence of environmental factors on medicinal plant quality and yield

2.6.1 pH-stress

Potential hydrogen (pH) plays a major role on plant growth in the soil by influencing the availability of various nutrients for plant uptake (Peterson, 1983; Kunh et al., 1995; Marschner, 1995). According to Brady and Weil (2008), elements such as calcium, magnesium, zinc, and copper are less available at pH 5 and below in the soil. This influences the enzyme actions, which in turn affect certain metabolic processes, and plant growth (Stern, 2006; Koehorst et al., 2010). As the pH approaches 7.5 and above, phosphorus, iron, manganese, boron, and zinc were less available for plant uptake in a study done on *Artemisia afra* L (Brad and Weil, 2008; Koehorst et al., 2010). The pH can be easily manipulated on the hydroponic system; Hydrogen Chloride (HCL) is used to lower pH, and sodium hydroxide (NaOH) to raise the pH. It has been scientifically proven that the availability of nutrients in plant growth medium affects secondary metabolite production, which may, subsequently, influence the medicinal properties of extracts derived from these plants (Cowan, 1999; Van Alstyne & Pelletrean, 2000; Economakis et al., 2002; Maggini et al., 2002; Sugumaran et al., 2013). Despite the fact that plants possess different medicinal properties, growing conditions are the key factors that affect their metabolism (Lin et al., 2006).

2.6.2 Light

Sunlight is an inexorable resource for photosynthesis in plants for production of carbohydrates and starch. The sunlight directly influences plant growth and flowering time. In the greenhouse, light irradiance changes affect plant productivity, physiology and cellular biochemistry notwithstanding other microclimates temperature, humidity and CO_2 concentration. The normal range of light intensity can improve the yield and quality of medicinal plants. Although in low light intensity, plants tend to produce low yield. Limited light can also change environmental factors such as temperature, air, carbon dioxide (CO_2), which are important for plant growth (Song et al., 2012).

2.6.3 Water stress

Drought has a crucial impact on the agricultural sectors as it threatens plant growth and productivity. However, prolonged water deficit may result in accumulation of secondary metabolites as essential compounds; thereby affecting quality of fruit and medicinal value. For example, a chemical compound, such as ascorbic acid (Vitamin C), obtained in orange fruit, play a major role in scavenging reactive oxygen species (ROS) in plants under water stress (Jiang and Zhang, 2002; Stevens et al., 2008). There is scientific evidence that illustrates secondary metabolite compounds induced by drought, such as white lupine, result in an osmotic adjustment. Therefore, strategic and deliberate implementation of water deficit by prolongation of watering intervals during crop cultivation can help mankind to achieve good quality crops. Under conditions of high light intensity, plants produce high starch and carbohydrate contents that contribute to their mass (Kose, 2014). However, when they are simultaneously exposed to more than one stress factor, the responses are not straightforward; for instance, plants exposed to light and drought stresses invest more in shoot and leaf production than root production (Guo et al., 2012). Prider & Facelli (2004) further argued that plants that are adapted to low light are sensitive to limited water supply. Hence, their large proportion of biosynthesis production meant for biomass, is allocated to light-capturing organs and this tend to create larger transpiration areas. The interaction between shade and water stress can affect plant growth as was observed in a study by Liu et al. (2007), whereby *Abutilou theoprasti* L. maintained sensitivity to variation in enzyme activities, which then contributed to stem elongation. The shade can reduce the impact of drought by limiting the loss of water in soil during evaporation (Holmgren, 2000; Guo et al., 2013). When plants are

exposed to drought stress, protection against oxidative damages is provided by both enzymatic and non-enzymatic mechanisms, which increase the concentration of antioxidant (Caser et al., 2016). The accumulation of such biosynthesised constituents in plants contributes to medicinal values that benefit human health (Lubbe & Verpole, 2011). The environmental factors play significant roles in the biosynthesis of secondary metabolites accumulation and enzyme activities; therefore, understanding these environmental factors will help us to understand the dynamics of plant biomass, morphological changes and physiological mechanisms involved in plants' immune responses (Caser et al., 2016).

CHAPTER THREE

The effects of light intensities varying pH and watering intervals on growth, antioxidant content-and -capacity of *T. violacea* cultivated hydroponically under greenhouse conditions

Abstract

Environmental stress factors such as high or low soil pH, water deficit, high temperature and shade may result in accumulation of reactive oxygen species in plants, which in turn may cause oxidative stress when in excess. This study was divided into two separate preliminary experiments. Simultaneous assessments of the effects of i) varied pH levels (pH 4, pH 6, pH 8) and ii) low light intensity and normal light intensity on plant growth, antioxidant-content and -capacity of extracts of *T. violacea*. The objectives of this exploratory preliminary study were to assess the effects of abiotic environmental stresses (pH, normal light and low light) on the antioxidant-content and -capacity of *T. violacea* under greenhouse conditions. Observations were recorded on the following parameters: plant height, plant fresh and dry weights, antioxidant-content and -capacity. Plant height was recorded weekly, and at two-month post-treatment, plants were harvested and fresh and dry weights were recorded. Dried aerial and root parts were analysed for antioxidant-content and -capacity. Results obtained from the first part of the study showed that plants exposed to pH 6 showed a marked increase in plant height (from 25-37 cm) after 2 months of treatment; although, generally, the variations in growth parameters among the different pH treatments were not significant ($p > 0.05$). Antioxidant-content and -capacity were not significantly different ($p > 0.05$) when pH treatments were compared. However, a high polyphenol content value (3 mg/g) was found in leaves of plants exposed to pH 8. Flavonol content was 2.3 mg/g in the roots exposed to pH 4. Overall, there was no significant difference ($p > 0.05$) in antioxidant-content and -capacity when pH treatments were compared. On the light experiment, it was observed that decreasing light intensity from normal to low light led to the elongation of plant height from 34.6-26.5 cm, as well as lower weight gain in the roots and leaves of plants. Higher leaf polyphenol content was obtained in plants grown under normal light intensity and those exposed to low light intensity. The polyphenol and flavonol content fluctuated between the averages of 5.8 ± 0.2 mg/g to 8.5 ± 0.3 mg/g. In conclusion, besides flavonol, increased leaf antioxidant-content and -capacity were produced by exposing *T. violacea* to normal light intensity. Meanwhile, pH 8 plants, in general, had increased antioxidant-content and -capacity levels.

3.1 Introduction

Mankind has relied on plants for centuries as primary sources for food and medicine (Van Wyk et al., 2009). In South Africa, it is estimated that about 200 000 indigenous traditional healers utilise indigenous plants for traditional medicine, and approximately 60% of the South African population consult these healers for their healthcare needs. Traditional healers and farmers believe that materials that are harvested from wild plants have more healing properties when compared to cultivated plants (Luseba et al., 2007). Consequently, the rate of overexploitation of medicinal plants in the wild has increased over recent years. It is estimated that between 4,000 and 10,000 wild populations of medicinal plants are endangered due to over-harvesting (Van Wyk et al., 2009). Some medicinal plants are harvested for their antioxidant and antimicrobial properties (Hutching, 1989; Zschocke et al., 2000; Taylor & Van Staden, 2001; Katerere & Eloff, 2008). For example, *Artemesia afra* is used to treat fever and cough in most parts of the Eastern Cape in South Africa (Wyk et al., 2009).

Continuous harvesting of parts of plants puts many species at risk of extinction. Therefore, there is an urgent need to protect wild medicinal plant species. The regulation of harvesting of wild medicinal plants through legislation is a strategy that is used by many governments to curb destruction of wild plant species. Other interesting approaches that are gaining traction are the use of high-tech cultivation methods such as tissue culture and hydroponics. Although these methods were mostly used for propagation and cultivation of food crops and ornamentals, these technologies could be used to enhance the propagation and cultivation of medicinal plants, which could offer an opportunity to achieve high quality production, optimise yield and achieve uniformity (Peter et al., 2005).

Hydroponics is an advanced cultivation method whereby medicinal plants can be cultivated in a controlled environment such as a greenhouse for commercial production. A hydroponic system is one of the biotechnology strategies used to conserve plants and manipulate secondary metabolites (Ncube et al., 2011). Peter et al. (2005) indicated that growing plants in a controlled environmental condition could lead to the manipulation of phenotypic variation of the concentrations of biological compounds being produced. Furthermore, when plants are grown under certain stresses, they tend to adapt and produce more secondary metabolites for their protection. For example, drought stress (proline), infection (flavonoids) and herbivores (alkaloids) (Peter et al., 2005). Some of these metabolites have antioxidant

properties. Antioxidant compounds produced by plants during stress conditions are very useful against free radicals that predispose humans to sickness and diseases. Antioxidants found in plant extracts can prevent free radicals from damaging tissue or organs in the body (Adewusi et al., 2011). Pietta (1998) defines oxidation as the transfer of electrons from one atom to another and represents an essential part of aerobic life and our metabolism. The phenolic compounds associated with antioxidant activity play a significant role in adsorbing and neutralizing free radicals (Zheng and Wang, 2001).

Free radicals are described as atoms that contain more than one unpaired electron (Daniels et al., 2015). In a human body, the unpaired electrons can react with oxygen to form superoxides that might be harmful to human tissue. Unhealthy diets such as spicy junk food, smoking of cigarettes and exposure to atmospheric pollutants can create more free radicals in the body. When the body is exposed to high levels of free radicals, the antioxidants that protect the immune system from diseases can decrease. Free radicals are associated with pathophysiological ailments such as atherosclerosis, arthritis, and ischemia, reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDs (Cook and Samman, 1996; Kumpulainen and Salonen, 1999). There is a huge interest in finding potential medicinal plants that can reduce the effects of free radicals on tissue injury in the body (Schuler, 1990). *Tulbaghia violacea* is one of the well-known medicinal plants in South Africa.

While many studies have shown that crude extracts of *T. violacea* have great medicinal potential against the nematode *Caenothabditis elegans,* and fungal and bacterial pathogens (McGawet al., 2000; Zheng and Wang, 2001; Naidoo et al., 2008; Soyingbe et al., 2013), there is somewhat inadequate information on antioxidant activities of *T. violacea*. The leaves of *T. violacea* (specimens found in the Transkei and KwaZulu-Natal areas) are consumed as a substitute for spinach and are a rich source of micronutrients (Aremu and Van Staden, 2013). Micronutrients such as Vitamin C, Vitamin E, Boron, and beta-carotene in diets contribute to the scavenging and reduction of free radicals (Lin et al., 1999; Opoku et al., 2000). Thus, *T. violacea* is a potential source of antioxidants.

Plants possess different antioxidant properties and contents and the growing conditions of plants have a strong bearing on the metabolism of antioxidants (Lin et al., 2006). According to Daniels et al. (2015) and McChesney (1999) environmental stress factors such as high or low soil pH, water deficit, high temperature, and shade may result in the accumulation of

reactive oxygen species in plants which in turn may cause oxidative stress when in excess. Although *T. violacea* is classified as a 'Least concern' in the Red list database in South Africa, the need to optimise its desirable pharmacological properties warrants the search for optimum cultivation practices for this species (Olorunnisola et al., 2011). The objectives of this exploratory preliminary study were to assess the effects of abiotic environmental factors (pH and light) on the antioxidant-content and -capacity of *T. violacea* plants under greenhouse conditions.

3.2 Materials and methods

3.2.1 Plant material

One-month old *T. violacea* seedlings supplied by Best Western Seedlings Nursery (VarkensVlei Road, Phillip, Western Cape, 7785, South Africa) were used in this experiment. Seedlings were propagated by dividing larger clumps of seedlings. The separated offsets were gently washed under running tap water for 5 min, and thereafter transplanted into 15 cm black plastic pots (Plastic for Africa PTY/LTD, Somerset West, Cape Town, 7130) filled with sterile river sand obtained from the Somerset river.

3.2.2 Greenhouse experimental design

The experiment was conducted at the Cape Peninsula University of Technology, Bellville, Western Cape, South Africa S33° 54' 0, E18° 38' 0 from February to April 2016. It was undertaken in a controlled environment (greenhouse structure), with maintained temperature between 24 – 26 °C during the day and 15 – 20 °C during night. The average humidity was 74%. Two separated preliminary experiments were carried out simultaneously. These were done to assess the effects of varying pH levels (4, 6 and 8), and the light intensity measured ranged from 300 lux to 500 lux on the growth and antioxidant-content and -capacity of *T. violacea*. The individually potted seedlings were placed on a cement floor inside a research greenhouse and spaced 30 cm apart. Plants were selected randomly and placed into the two major experimental groups: pH and light intensity (normal light intensity and low light intensity). The six-week old propagated plants were then exposed to the varied environmental treatments (Figure 3.1).

Experiment 1: Plants were exposed to low, moderate and high pH levels (4, 6 and 8). The pH 4, pH 6 and pH 8 levels were achieved by using hydrochloric acid to lower the pH and sodium hydroxide to raise pH in the plant nutrient solution and monitored using a JENCO vision plus instrument. The nutrient solution applications to all experimental plants were supplied by means of hydroponics using the drip irrigation system. Plants were irrigated with Nutrifeed fertilizer (Starke Ayres, Cape Town) containing the following ingredients: N (65 mg/kg), P (27 mg/kg), K (130 mg/kg), Ca (70 mg/kg), Cu (20 mg/kg), Fe (1500 mg/kg), Mo (10 mg/kg), Mg (22 mg/kg), Mn (240 mg/kg), S (75 mg/kg), B (240 mg/kg) and Zn (240 mg/kg). The nutrient solution was prepared by dissolving 60 g of fertilizer into a 60 L black reservoir filled with tap water. Airstones were placed in each of the reservoirs to add oxygen to the nutrient solution. The plants were then subjected to a three-day watering interval meaning they received water after 3-days. The effects of light intensity and pH on growth, and antioxidant-content and -capacity were assessed on the tested plants.

Experiment 2: Plants were exposed to low and normal light intensity. Normal light intensity is regarded as the light entering the greenhouse through the greenhouse covering material (white corrugated polycarbonate). The low light intensity was achieved by covering the plants in the same greenhouse with 40% shadenet (Allnet, Epping Industria). During the study period, from February to April (2 month), the average light intensity range was 300-500 lux during day. In addition, plants were exposed to low watering regime (watering interval of 30 days)

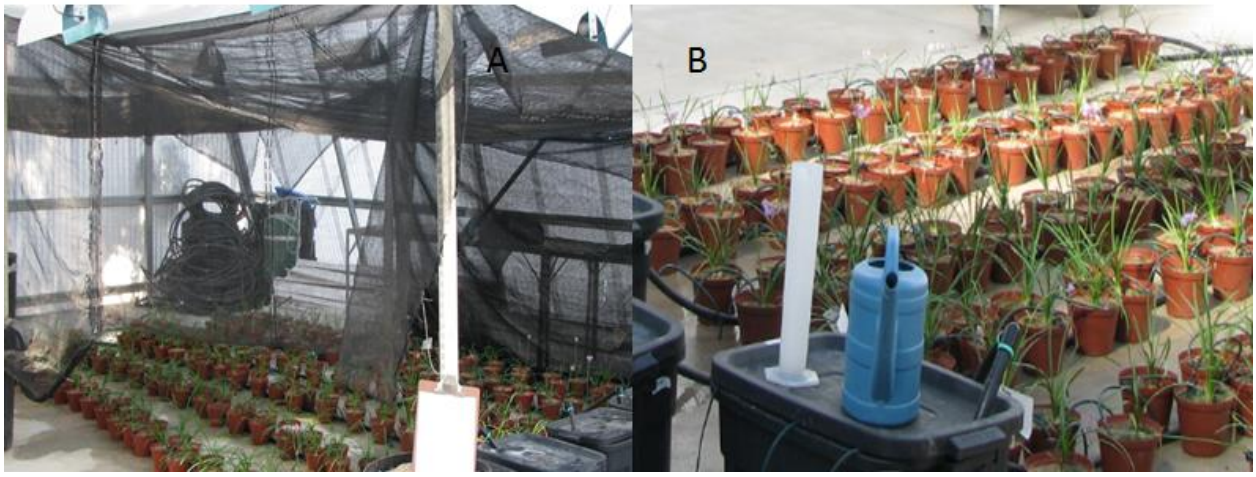

Figure 3.1: Setup of the exposure of *T. violacea* under A) low light intensity B) normal light intensity

3.2.3 Data collection

The height of the plant was measured at weekly intervals for two months using a measuring tape. The measurements were recorded from the river sand substrate level to the tip of the tallest shoot. In order to determine plant biomass, the mature plants (two months post-treatment) were harvested and weighed for their fresh weight at the end of the experiment, after which they were dried in the thermo-oven at 40 °C for 7-14 days and dry weights were captured.

3.2.4 The antioxidant analysis

For the antioxidant analysis harvested materials were immediately dried in a fan-drying laboratory oven (Oxidative Stress Research Centre, Faculty of Health and Wellness Sciences at CPUT, Bellville, South Africa) at 40 °C for 7-14 days. The dried plants were then separated into leaves and bulbs and ground into fine powder using a Junkel and Kunkel model A 10 mill. The ground powder was then stored into air-tight stopper glassware prior to analyses. To obtain crude extract, the finely ground leaf and bulb materials of this plant was then stirred separately in ethyl alcohol (EtOH) (Saarchem, South Africa), and thereafter centrifuged at 4000 rpm for 5 min (Daniels et al., 2015). The crude extract of this species was used for the below-mentioned chemical analyses.

3.2.4.1 Determination of polyphenol, flavonol and flavanone contents

The Folin Ciocalteu method was used to determine the total polyphenol content of the various crude extracts (Singleton et al., 1974 and Swain and Hills, 1959). Twenty-five microliter of sample was mixed with 125 µLFolin–Ciocalteu reagent (Merck, South Africa), diluted 1:10 with distilled water and after 5 min., 100 µL (7.5%) aqueous sodium carbonate (Na_2CO_3) (Sigma-Aldrich, South Africa) was added to wells of a 96-well microplate. The plates was incubated for 2 h at room temperature and the absorbance was read at 765 nm using a Multiskan plate reader (Thermo Electron Corporation, USA). The standard curve was prepared using 0, 20, 50, 100, 250 and 500 mg/L gallic acid in 10% EtOH and the results were expressed as mg gallic acid equivalents per g dry weight (mg GAE/g DW).

The flavonol content was determined using quercetin 0, 5, 10, 20, 40, and 80 mg/L in 95% ethanol (Sigma-Aldrich, South Africa) as standard. For each sample well, 12.5 µL of the crude sample extracts was mixed with 12.5 µL 0.1% HCl (Merck, South Africa) in 95% ethanol, 225 µL 2% HCl and incubated for 30 min at room temperature. The absorbance was read at 360 nm, at a temperature of 25 °C (Mazza et al., 1999). The results were expressed as mg quercetin equivalent per g dry weight (mg QE/g DW).

To determine the flavanone content 100 µL of sample was mixed with 200 µL 1% 2.4-dinitrophenylhydrazine (DNPH) (2% H_2SO_4 in methanol (MeOH). After incubation at 50 °C for 50 min., 700 µL of 10% Potassium hydroxide (KOH) in 70% MeOH was added (Kosalek et al., 2004). The samples were centrifuged and 30 µL of the resulting supernatant mixed with 270 µL MeOH in a 96-well plate and the absorbance read at 495 nm. A linear standard curve using 0, 0.2, 0.5, 1.0, 1.5, and 2.0 mg/mL naringenin (Sigma-Aldrich, South Africa) in methanol was included. The results were expressed as mg naringenin equivalent per g dry weight (mg NE/g DW).

3.2.4.2 Determination of antioxidant capacity (FRAP, ABTS, ORAC)

The FRAP assay was performed using the method of Benzie and Strain (1999). In a 96-well microplate, 10 µL of the crude sample extract was mixed with 300 µL FRAP reagent [0.3 M acetate buffer, pH 3.6 (Saarchem, South Africa), 10 mM 2,4,6-tripyridyl-*s*-triazine (TPTZ) in 0.1 M HCl (Sigma-Aldrich, South Africa), 20 mM Iron (III) chloride hexahydrate ($FeCl3{\cdot}6H_2O$) (Sigma-Aldrich, South Africa), 6.6 mL distilled water and incubated for 30 min. at 37 °C in the plate reader. Absorbance was measured at 593 nm. L-ascorbic acid (Sigma-Aldrich, South Africa) was used as a standard with concentrations varying between 0 and 1000 µM. The results were expressed as µM ascorbic acid equivalent per g dry weight (µM AAE/g DW).

The ABTS assay was performed following the method of (Re et al., 1999). The stock solutions included a 7 mM ABTS and 140 mM Potassium–peroxodisulphate ($K_2S_2O_8$) (Merck, South Africa) solution. The working solution was then prepared by adding 88 µL $K_2S_2O_8$ to 5 mL ABTS solution. The two solutions were mixed well and allowed to react for 24 h at room temperature in the dark. Trolox (6-Hydrox-2,5,7,8-tetramethylchroman-2-

carboxylic acid) was used as the standard with concentrations ranging between 0 and 500 µM. Crude sample extracts (25 µL) were allowed to react with 300 µL ABTS in the dark at room temperature for 30 min before the absorbance was read at 734 nm at 25 °C in a plate reader. The results were expressed as µM/Trolox equivalent per g dry weight (µM TE/g DW).

The peroxyl radical was generated using 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH) (Sigma-Aldrich, South Africa), prepared fresh for each determination according to the method of (Prior et al., 2003). Fluorescein was used as the substrate. Absorbance was read using a Fluorskan Ascent plate reader (Thermo Electron Corporation, USA) with the fluorescence conditions set at 485 nm excitation and 530 nm emissions. The standard curve was linear between 0 and 25 µM/Trolox. The results were expressed as µM/Trolox equivalent per g dry weight (µM TE/g DW).

3.2.5 Statistical analysis

The statistical significance among antioxidant activity values of the various crude plant extracts was determined using one-way analysis of variance (Anova) where $P < 0.05$ was considered statistically significant. Means were separated using the posthoc Tukey test. The computer program employed for the statistical analysis was Medcalc version 9.4.2.0 (Medcalc, Belgium). Microsoft Office Excel 2006, version12.0.6214.1000 (Microsoft Corporation, USA) was employed to determine the correlation between antioxidant contents and activity.

3.5 Results

3.5.1 Plant growth (height, fresh and dry weight)

Light intensity

The results obtained in this study indicated that light intensity had a significant affect ($p < 0.05$) on the vegetative growth of this species (Figure 3.2a). Higher mean shoot lengths (28-34.6 cm) were obtained under low light compared to normal light intensity throughout the duration of the study evidencing that shoot length significantly increased with decreasing of light intensity. Even though leaf area was not measured, it was observed that leaves in low light intensity were narrower than those under normal light were broader.

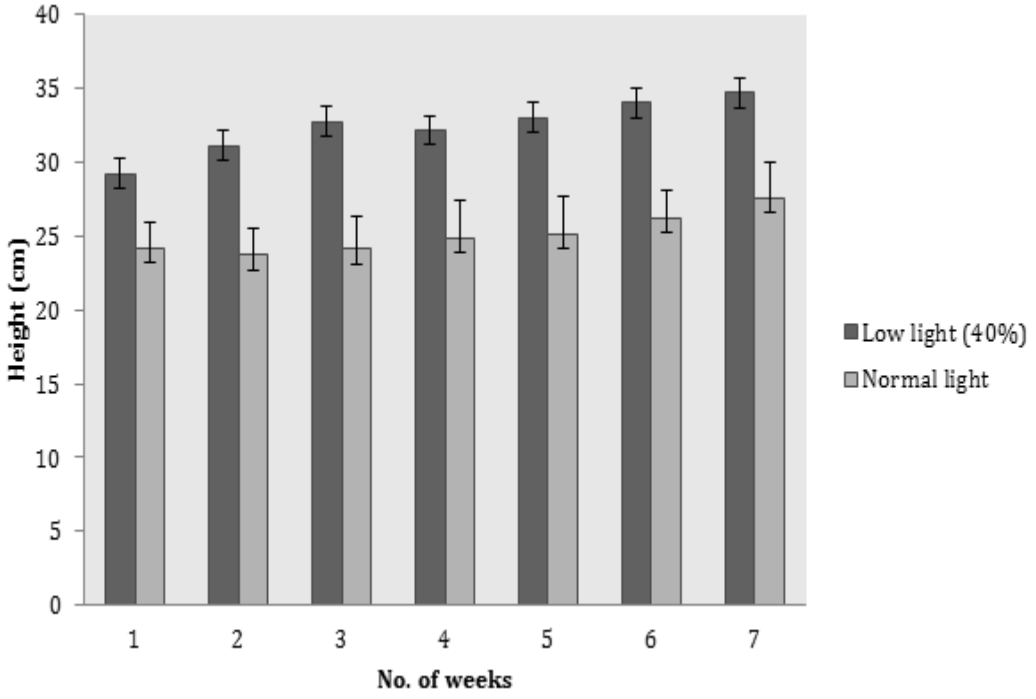

Figure 3. 2a: Effects of low or normal light intensity (watered after 30 days) on the height of *T. violacea* at 1-7 weeks.

pH levels

There was no significant difference ($p > 0.05$) in heights when pH levels were compared. Similar heights ranging from 25-27 cm during the 1st week to 3rd week were recorded. However, pH 6 recorded moderately higher heights from the 4th week to last week of the experiment. The highest mean height value 44 cm of *T. violacea* was obtained under pH 6 level (Figure 3.2b).

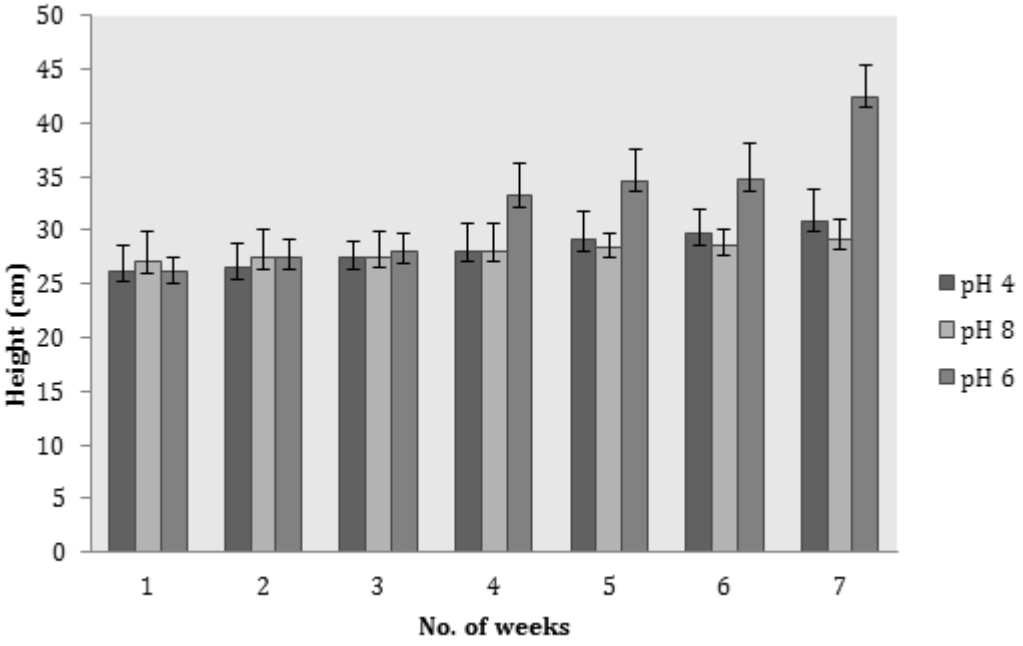

Figure 3.2b: Effects of varying pH levels on the height of *T. violacea* at 1-7 weeks.

3.5.2 Fresh & dry weight

Light intensity

For the fresh weight, under low light exposure, plants were heavier than normal light ($p < 0.05$). The opposite was true for the dry weight (2.4 g), which was higher (13 g) under exposure to normal light compared to low light (8 g) (Figure 3.3b); although there was no significant difference ($p > 0.05$) between the two light intensities tested.

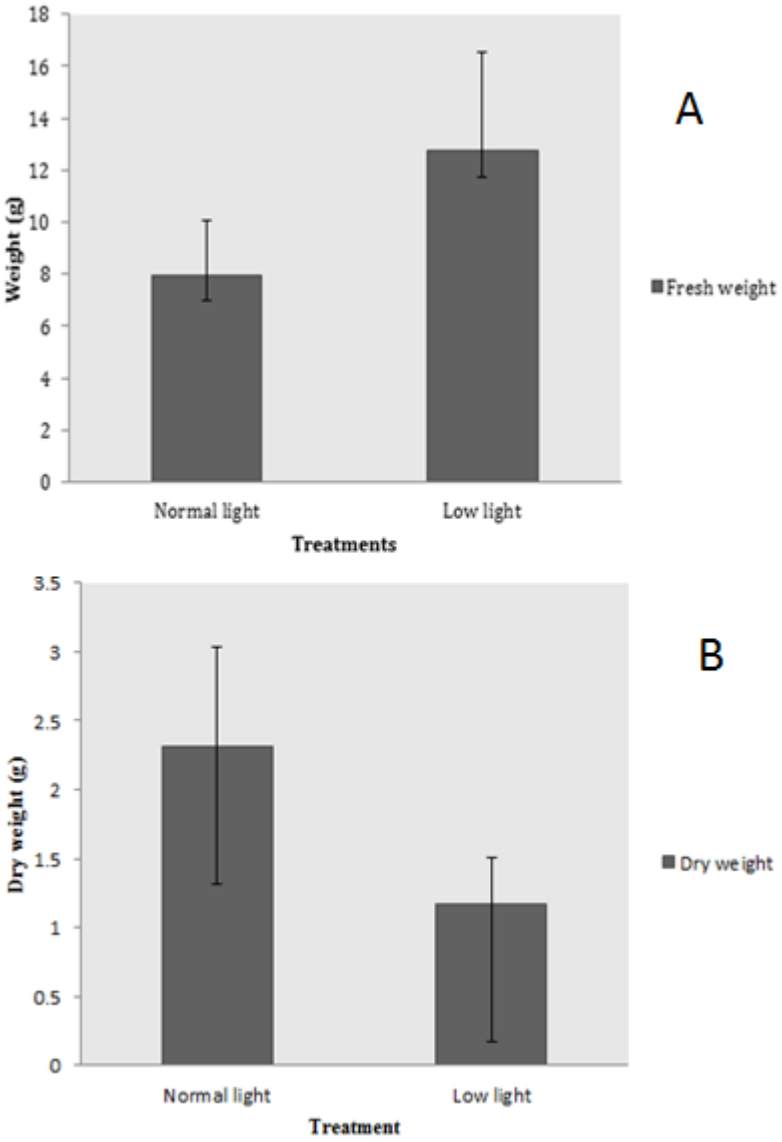

Figure 3.3a: Effects of low or normal light intensity on the fresh (A) and dry weight (B) of *T. violacea.*

pH levels

The weights of plants under different pH levels were measured in this study. The highest mean fresh weight (67.5 g) was observed in plants grown under pH 4 treatment (Figure 3.3b). Generally, the fresh weights decreased significantly ($y = -0.1599x + 14.849$; $r^2 = 0.9$) with increasing pH level (Figure 3.3b: B). When the pH level was 8, both fresh and dry weights of *T. violacea* showed significant decreases compared to those at lower pH levels (Figure 3.3b).

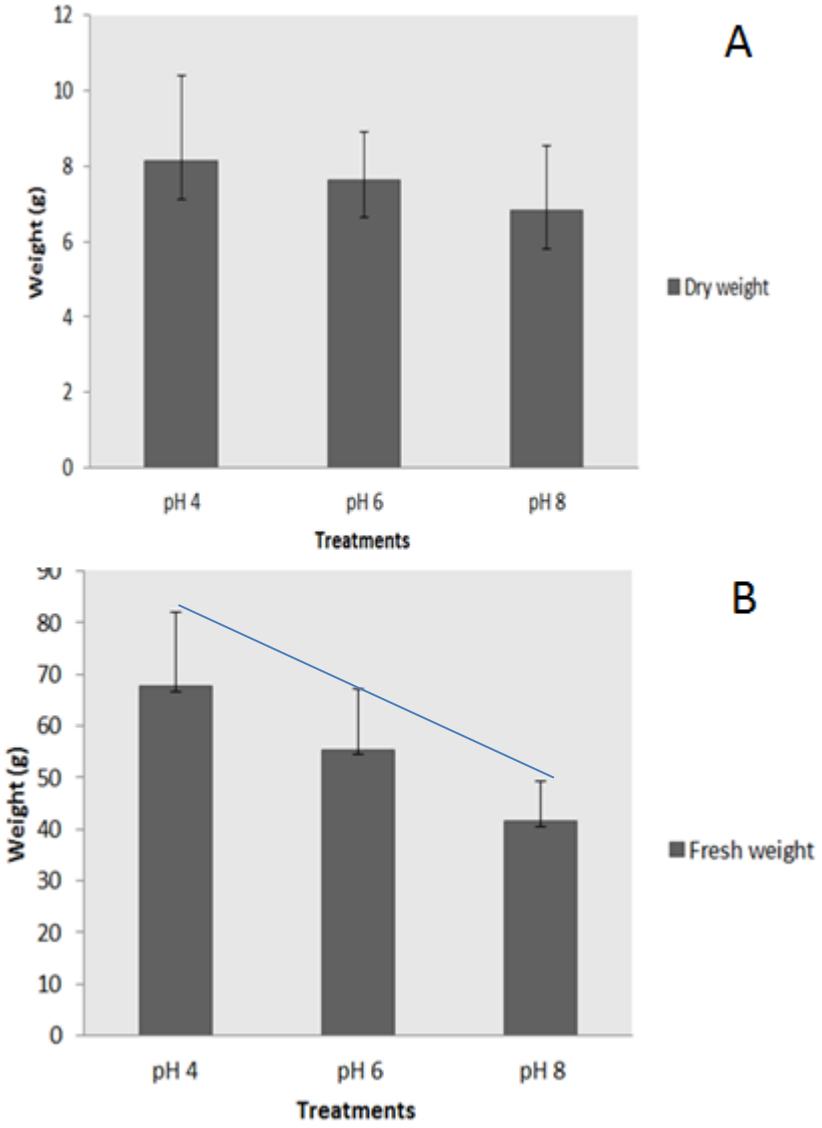

Figure 3.3b: Effects of varied pH levels on the dry weight (A) and fresh weight (B) of *T. violacea*.

3.5.3 Antioxidants content

Light intensity

When plants were subjected to the two light intensities, their antioxidant-content and -capacity fluctuated in the leaves and bulbs of *T. violacea*. The polyphenol contents of the leaves ranged from 5.8 mg/g to 8.5 mg/g (Figure 3.4a), and this was significantly higher (df, 2,9; $F = 5.3$; $p < 0.05$) in normal light-exposed plants than in the low light-exposed counterparts. In the roots, however, polyphenol content was rather higher in the low light treated plants than those exposed to normal light.

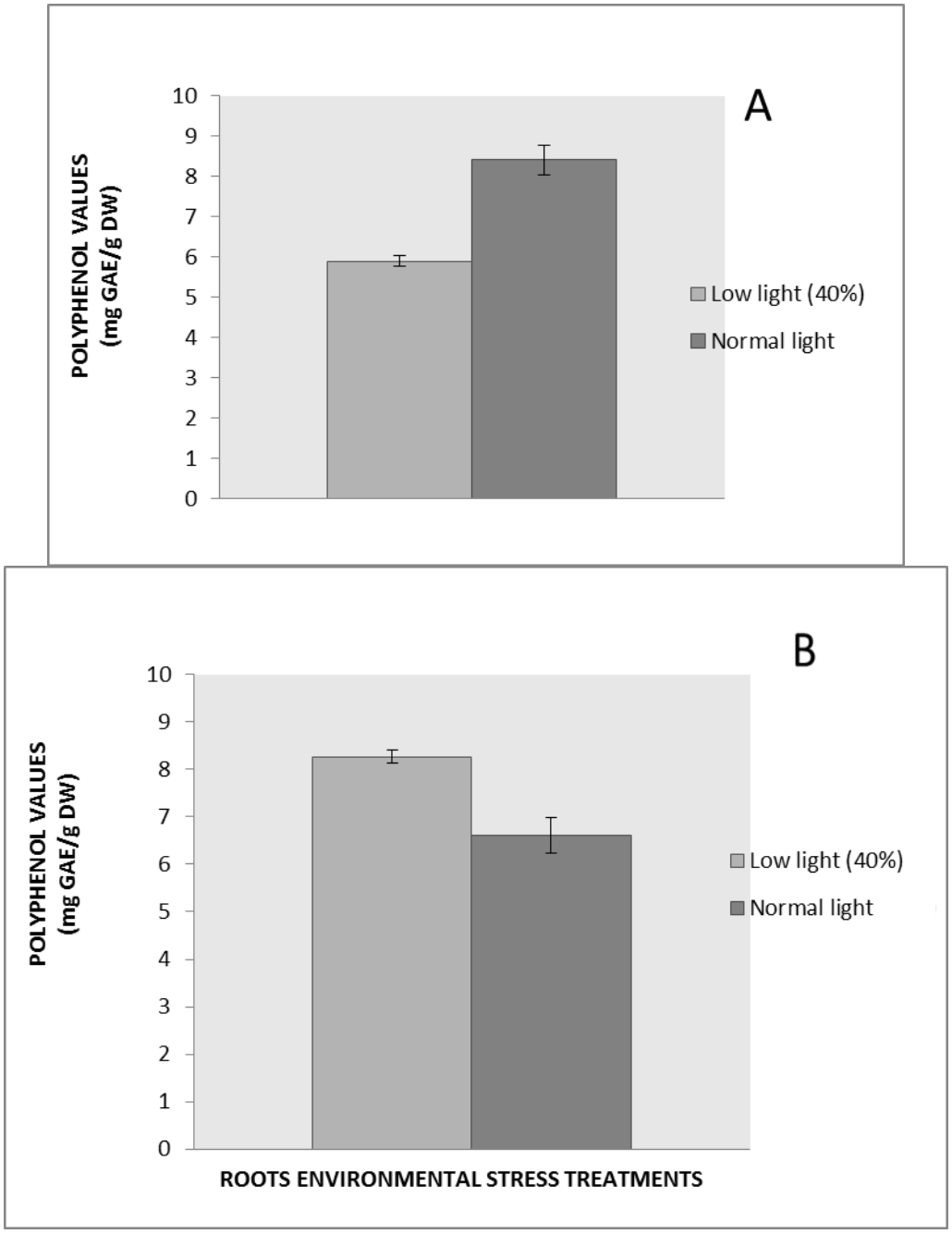

Figure 3.4a: The total polyphenol (mg GAE/g dry weight) content of the leaves and roots of *T. violacea* plants under different light intensities. Values represent the means ± SD for the leaves (A) and roots (B) (n = 10).

pH levels

The polyphenol content of *T. violacea* leaves was significantly increased at the highest pH (5.2 mg/g) compared to the lower pH treatments (Figure 3.4b) in the leaf. Generally, polyphenol content was higher in the roots than in the leaves for all the pH level tested.

However, there was no significant ($p > 0.05$) variation in root polyphenol contents among treatments.

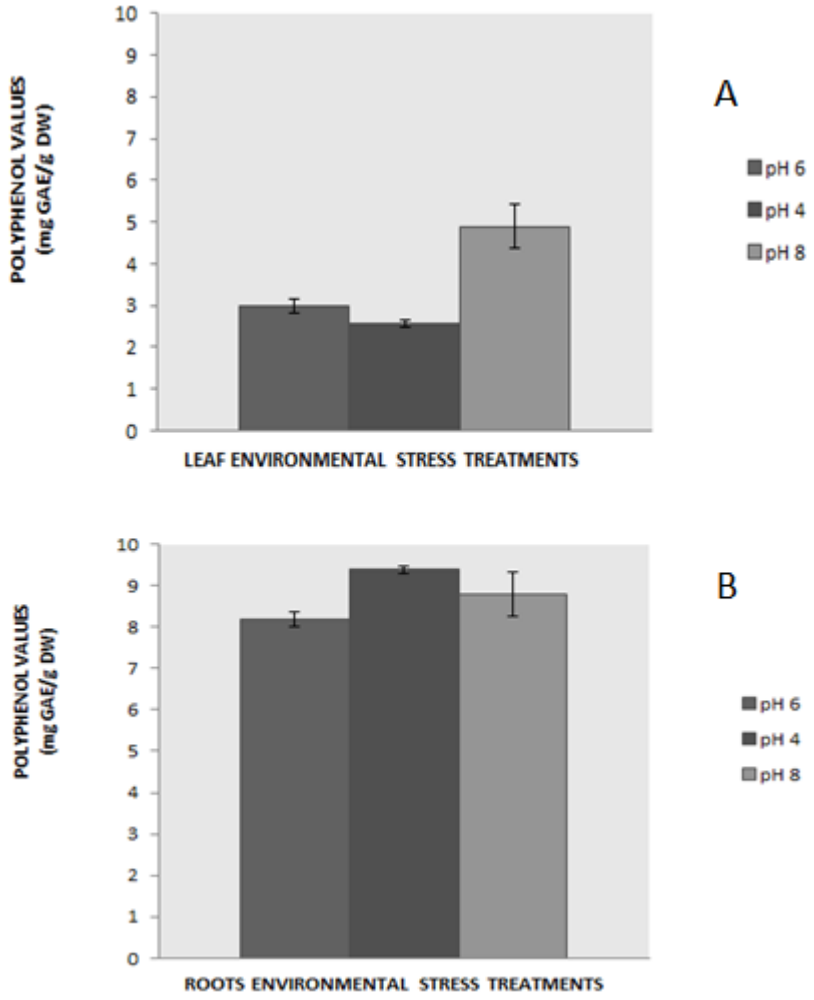

Figure 3.4b: The total polyphenol (mg GAE/g dry weight) content of the leaves and roots of *T. violacea* plants under varied pH levels. Values represent the means ± SD for the leaves (A) and roots (B) (n = 10).

Light intensity

The flavonol content in the leaves did not vary significantly (df, 2, 9; $F=4.8$; $p > 0.05$) when low and normal light intensities were compared (Figure 3.5a), despite the higher mean value of flavonol content (2.52 ± 0.1 mg/g) obtained for plants grown under normal light intensity. The roots had a significantly reduced flavonol content compared to the leaves.

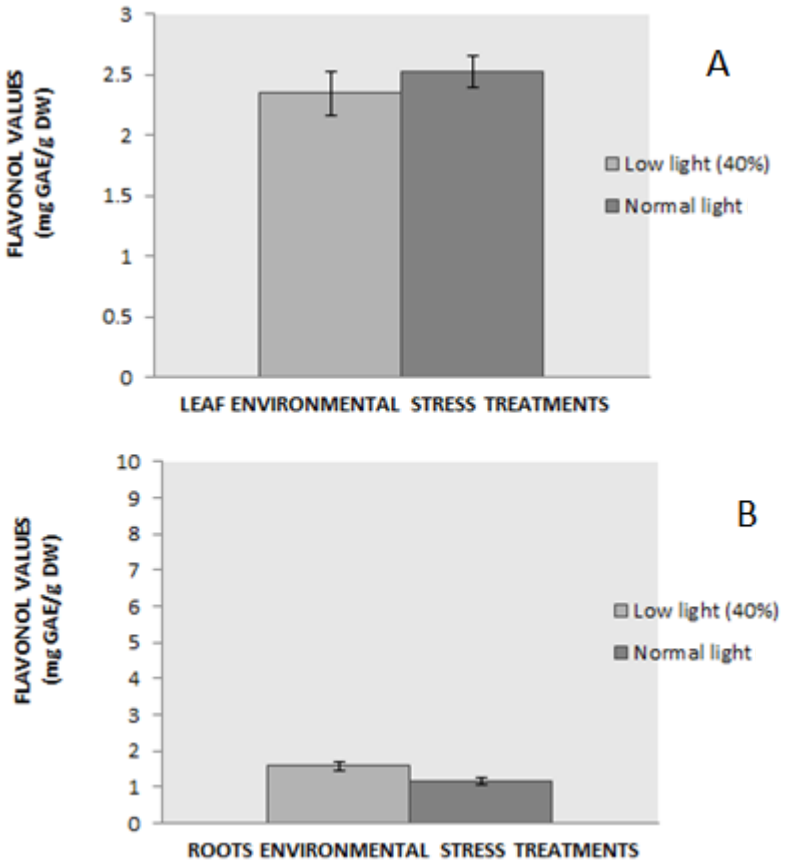

Figure 3.5a: The total flavonol (mg GAE/g dry weight) content of the leaves and roots of *T. violacea* plants under different light intensities. Values represent the means ± SD for the leaves (A) and roots (B) (n = 10).

pH level

The flavonol contents were significantly higher at higher pH levels (6; 2.3 mg/g and 8; 2 mg/g) than at the lowest pH level 4 (1.5 mg/g) in the leaves of *T. violacea* (Figure 3.5b), and a similar trend was observed for the roots; although roots had a much lower value for flavonol content than the leaves.

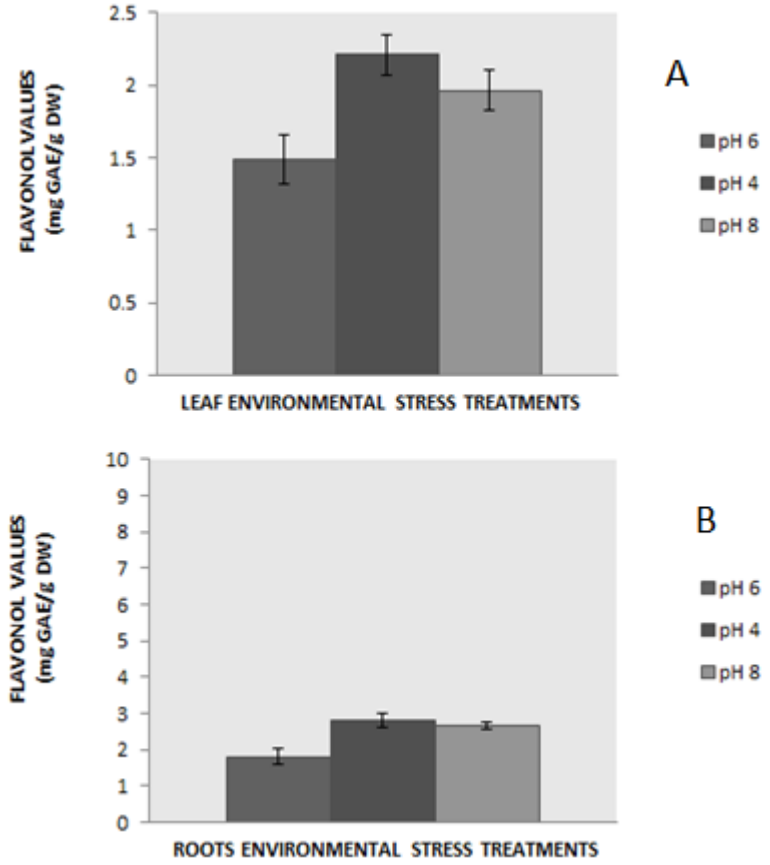

Figure 3.5b: The total flavonol (mg GAE/g dry weight) content of the leaves and roots of *T. violacea* plants under varied pH levels. Values represent the means ± SD for the leaves (A) and roots (B) (n = 10).

3.5.4 Antioxidant capacity

Light intensity

Plants exposed to normal light intensity showed significantly higher antioxidant activity FRAP value (12 mg/g) in the leaves ($p < 0.05$) when compared to low light for the aerial part (Figure 3.6a). However, the FRAP value was slightly lower among normal light-exposed plants than low light-exposed for the root material, and the difference was not significant ($p > 0.05$).

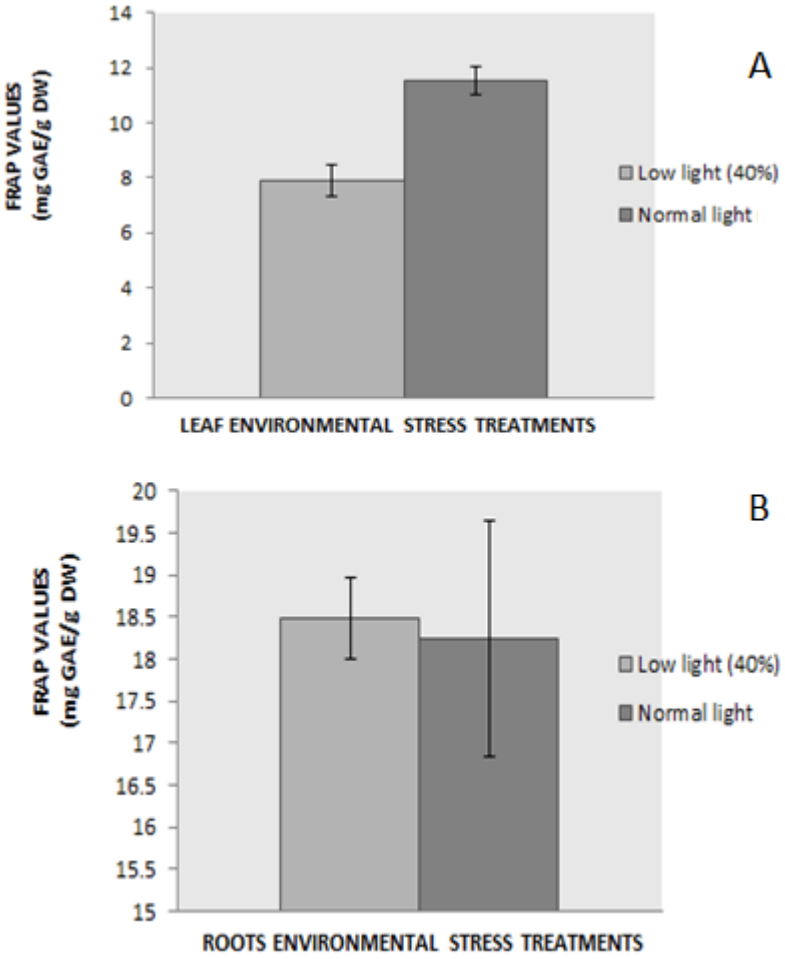

Figure 3.6a: The total FRAP (mg GAE/g dry weight), content of the leaves and roots of *T. violacea* plants under different light intensity. Values represent the means ± SD for the leaves (A) and roots (B) (n = 10).

pH levels

For the FRAP, capacity results indicated that pH 8 yielded a significantly higher value ($p < 0.05$) for the leaf parts compared to the other two lower pH levels. The FRAP did not vary significantly among pH treatment for the roots (Figure 3.6a). Generally, the FRAP values were higher in the roots than the aerial parts for all the three pH levels.

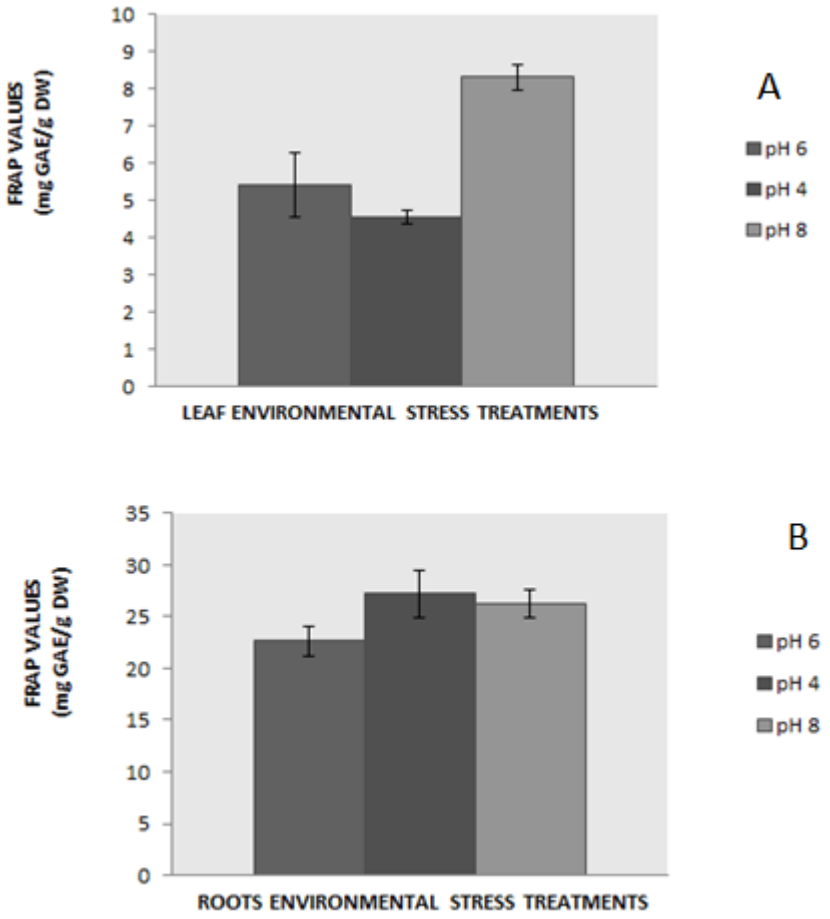

Figure 3.6b: The total FRAP (mg GAE/g dry weight) content of the leaves and roots of *T. violacea* plants under varied light intensity. Values represent the means ± SD for the leaves (A) and roots (B) (n = 10).

Light intensity

The ORAC value was significantly ($p < 0.05$) higher in the leaves of *T. violacea* after exposure of plants to normal light intensity. The ORAC values did not significantly vary between normal and low light intensities for the roots. The leaves had increased more ORAC values (ranged from 250-350 mg/g) than the roots (ranged from 100-250 mg/g) for both low and normal light 340 mg/g (Figure 3.6a).

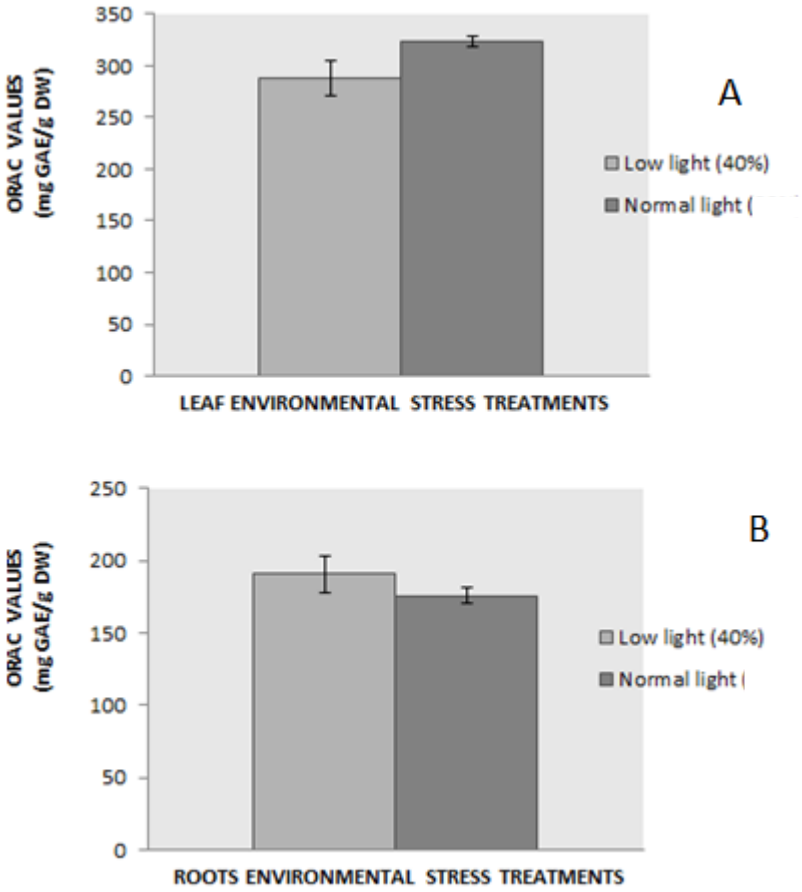

Figure 3.7a: The total ORAC (mg GAE/g dry weight) content of the leaves and roots of *T. violacea* plants under different light intensity. Values represent the means ± SD for the leaves (A) and roots (B) (n = 10).

pH levels

Overall, there was significant difference ($p < 0.05$) in ORAC values when the different pH treatments were compared for both roots and leaves. At pH 6 level, the ORAC value of leaf was 180 mg/g significantly higher when compared to pH 4 with the lowest value of 140 mg/g, while pH 8 level was significant higher ($p < 0.05$) compared to the other two lower pH treatments for the leaf materials (Figure 3.7b). For the root samples, the ORAC values obtained for plants exposed to pH 6 and pH 8 were similar.

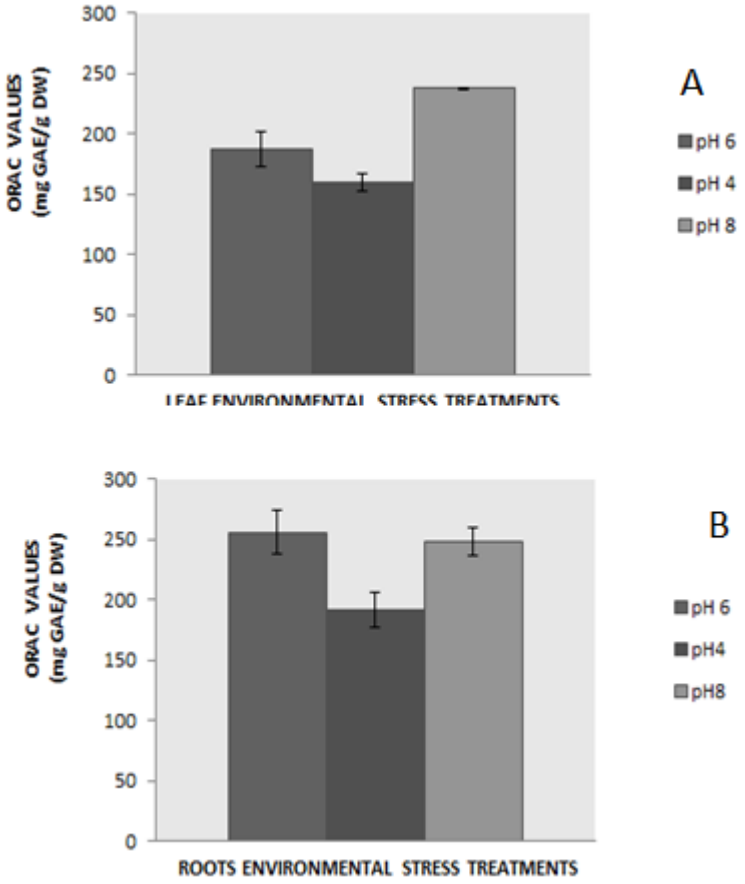

Figure 3.7b: The total ORAC (mg GAE/g dry weight) content of the leaves and roots of *T. violacea* plants under varied pH levels. Values represent the means ± SD for the leaves (A) and roots (B) (n = 10).

3.6 Discussion

3.6.1 Light intensity

Light is among the most important environmental factors that influences a plant's basic physiological processes, such as photosynthesis, respiration, transpiration and carbohydrates (Agaugu et al., 1995,). In this study, longer mean shoot length was obtained among plants under low light intensity compared to their plants under normal light intensity after 2 months, evidencing that shoot length significant increases with decreasing of light intensity. Low light intensity caused elongated internodes and long small leaves in *Zea mays* (Kubatsch and Gruneburg, 2007). At a low light intensity, a large concentration of carbohydrates are produced during photosynthesis to promote shoot elongation (Kubatsch and Gruneburg, 2007). However, plants exposed to normal light intensity had high dry and fresh weights than the low light intensity. Zervoudakis et al. (2012) reported similar results in a study involving

Salvia officinalis L., wherein plant height and leaf photosynthetic pigments increased at low light treated plants, whereas the plant dry mass, number of the leaves and physiological parameters showed a strong positive correlation with the light intensity. Kubatsch and Gruneburg (2007) demonstrated that a reduced light intensity significantly decreased fresh and dry weights of *Schefflera arboricola*, while high light increased fresh and dry weight. Plants grown under low light intensities have a decreased photosynthetic rate per leaf area, which is important for production of carbohydrates (Woledge, 1971). This explain why plants grown under low light had a significantly decreased dry weight compared to those grown under normal light intensity. Low light intensity decreases the photosynthetic rate, increases etiolation and defoliation (Kose, 2014). Other studies have shown that light intensity can influence leaf anatomy and morphology of plants, for example, Wilson and Cooper (1969) showed that Lolium plants grown in weaker light had much smaller stomata than those grown under stronger light conditions. They further argued that the effect of light intensity on photosynthesis could be explained in terms of changes in stomatal size, as well as possible influence of low carboxydismutase activity on stomatal aperture.
These results indicated that variation in light intensity significantly influenced the vegetative growth of this species, and consequently, yield of medicinal materials.

In this study, the effects of light on both antioxidants-content and -capacity in the leaves and bulbous roots of *T. violacea* were varied when subjected to normal light intensity. The polyphenol content were markedly superior in leaves of *T. violacea* at normal light intensity compared to low light intensity. Earlier studies on *Labisia pumila* revealed that total phenolic and flavonoid content, as well as antioxidant activity in three varieties, had consistently higher values for flavonol and polyphenol content as well as higher antioxidant activity when exposed to high irradiance (70% IR) over lower irradiance (Karimi et al., 2013). It is interesting to note that in the roots, while not significantly different, low irradiance induced higher antioxidant contents and activities. This finding corroborates that of Daniels et al. (2015), which found that the total polyphenol content was higher in the roots of *G. multifolia* subjected to low light intensity compared to high light intensity. The ORAC capacity was significantly affected by environmental factors in the current study. Both normal light and low light obtained the highest ORAC in the leaves. Generally, when plants are exposed to dry and normal light conditions, their roots penetrate deep into the soil in search of water and by active cellular molecules and biochemical pathways to modulate water transport and metabolism. The results obtained in this study corroborate those reported by Lin et al. (2006)

on the effects of drought in the leaves of sweet potatoes that resulted in high antioxidant activity. Chemical compounds, such as ascorbic acid (Vitamin C) obtained in orange fruit, play a major role in scavenging reactive oxygen species (ROS) in plants under water stress (Jiang and Zhang, 2002; Stevens et al., 2008). The low light resulted in significantly higher in ORAC values in the leaves of *T. violacea.*

3.6.2 pH

The highest mean fresh and dry weights were observed in plants grown at pH 4 compared to pH 6 and pH 8, respectively. Generally, in this study, the fresh and dry matter decreased significantly with increasing pH levels. This is consistent with the findings of Anugoolprasert et al. (2012) who reported that Sago palm grown at pH 4 tended to be heavier than those grown at pH 5.7. In an earlier experiment, Hoestra (1968) found good growth of apple seedlings at pH 3.8. The responses of plants to pH variations are influenced by genotype, species and growth medium.

While the effect of pH on growth showed clear patterns, the effect of pH on antioxidant activities of leaf and root yield varied results; however, pH 4 and pH 8 treatments significantly affected the FRAP levels in roots of *T. violacea* compared to pH 6 . Scientific literature on the effects of different pH levels on secondary metabolites of *T. violacea* is fairly limited and made comparisons to this study difficult. According to Daniels et al. (2015) and McChesney (1999), environmental stress factors, such as variation in soil pH, water deficit, high temperature, light, and shade may result in accumulation of reactive oxygen species in plants, which in turn may cause oxidative stress when in excess. Many previous studies have proved that reducing pH actually favours high production of secondary metabolites (Sáenz-Carbonel et al., 2001; Radić et al., 2016).

3.7 Conclusion

In conclusion, better accumulation of antioxidants in the leaves was evident, as well as higher antioxidant activity when *T. violacea* was cultivated under normal light intensity. In contrast, the roots showed higher accumulation of antioxidants and antioxidant activity for plants exposed to low light intensity. While plant heights was higher under low light, the corresponding fresh and dry weights were lower under low light compared to normal light intensity. The pH 8 was more effective in producing increased levels of antioxidants

(polyphenols) and antioxidant activity (FRAP and ORAC) in the leaves when compared to pH 4 and 6. Growth of plants (fresh and dry weights) reduced with increasing pH levels. Further research on the effect of environmental factors on plant growth and antifungal activity are further discussed in the following chapters.

CHAPTER FOUR

Effects of Light Intensities and Varying pH on Growth, Nutrient Uptake and Antifungal Activities of Hydroponic Cultivated *Tulbaghia violacea* L under Greenhouse Conditions

Abstract

In South Africa, *Tulbaghia violacea* is one of the most harvested plants in the wild for it is rich in medicinal properties. The demand for this species is very high and supply cannot meet current and future demands, thereby, warranting the search for high-yielding plant cultivation technologies. This study, therefore, is aimed to assess the interactive effects of light and pH on plant growth, nutrient uptake and antifungal of extracts of plants (*T. violacea*) grown hydroponically.

One-month old *T. violacea* plantlets were grown under two light intensities (low light and normal light) in a greenhouse and were simultaneously subjected to the following pH levels: pH 4, pH 6 or pH 8. Plant growth parameters, such as height, and fresh and dry weights were determined. Leaf samples were analysed for macro-and micro-nutrient contents. The plant extracts from the various treatments were tested in an antifungal bioassay (minimum inhibitory concentration [MIC]). Varied effects of different pH levels (4, 6 and 8) and light intensities (low and normal) on plant height, and fresh and dry weights were recorded. The results further revealed that there was a significant difference (df, 2, 57; $F = 12.63$; $p < 0.001$) in total dry weights. The highest dry weight was achieved under normal light intensity at pH 4 (8.29 ± 0.802 g). Significant interactive (df, 2; $F = 0.001$; $p < 0.001$) effects between pH and light on fresh and dry weights were observed. Extracts from plants grown under normal light intensity and at the highest acidic level (pH 4) showed superior antifungal activity, with MIC values of 0.18 ± 0 to 0.375 ± 0.04 mg/ml at 6 h and 1.5 ± 0 to 0.97 ± 0.18 mg/ml at 18 h. In conclusion, the interactive effects of pH and light intensity on the growth of *T. violacea* was demonstrated in this study, and growing *T. violacea* under normal light and acidic conditions favoured increased plant biomass and antifungal activities.

4.1 Introduction

Potential hydrogen (pH) plays a major role on plant growth in the soil by influencing the availability of various nutrients for plant uptake (Peterson, 1983; Kunh et al., 1995;

Marschner, 1995). According to Brady and Weil (2008), elements such as calcium, magnesium, zinc, and copper are less available at pH 5 and below. This influences the enzyme actions, which in turn affects certain metabolic processes, and plant growth (Stern, 2006; Koehorst et al., 2010). As the pH approaches 7.5 and above, phosphorus, iron, manganese, boron, and zinc were less available for the plant uptake in a study done on *Artemisia afra* L (Koehorst et al., 2010). The pH can be easily manipulated on the hydroponic system; Hydrogen chloride (HCl) is used to lower pH, and Sodium hydroxide (NaOH) to raise the pH. It has been scientifically proven that the availability of nutrients in a plant growth medium affects secondary metabolite production, which may influence the medicinal properties of extract derived from these plants (Economakis et al., 2002; Maggini et al., 2002; Sugumaran et al., 2013).

Despite the fact that plants possess different medicinal properties, growing conditions are among the key factors that affect plant metabolism (Lin et al., 2006). According to Daniels et al. (2015), environmental stress factors, such as variation in soil pH, water deficit, high temperature, light, and shade may result in accumulation of reactive oxygen species in plants, which in turn may cause oxidative stress when in excess. Even though some factors can be easily controlled, light is more difficult to control (Zhao et al., 2012). When the light changes, it affects plant morphology, physiology, and microstructure which may have an impact on the plant's production (Dai et al., 2009).

Plants require a certain intensity of light in order to achieve optimal growth and if the intensity of light is too high or too low, the photosynthetic rate will slow down (Zhao et al., 2012). When plants are subjected to shade, the light becomes limited, and light intensity can also change environmental factors, such as temperature, air, carbon dioxide (CO_2), which are important for plant growth (Song et al., 2012). When plants are simultaneously subjected to low light intensity and high nutrients, plants will allocate most of their photosynthetic end-product for growth processes, and this decreases the concentration of secondary metabolites (Bryant et al., 1983; Cronin and Hay, 1996). However, the concentration of secondary metabolites increases when plants are subjected to high light, which favours excessive accumulation of inorganic carbon compounds and less nutrient availability (Cronin and Hay, 1996). There is limited scientific literature on the effects of pH treatments under varying light intensities on nutrient uptake and antifungal activity of medicinal plants cultivated hydroponically in controlled environments.

T. violacea is commonly known as wild garlic, society garlic, sweet garlic, wildeknoffel (Afrikaans) and itswelelomlambo (Xhosa). It belongs to the family of Amaryllidaceae (Kubecetal., 2002; Harris, 2004). In South Africa, *T. violacea* bulbs and leaves are traditionally utilised for treatment of gastrointestinal ailments, asthma, fever, tuberculosis and the leaves are used to treat cancer of the esophagus (Kulkarni et al., 2005; Van Wyk et al., 2009). *Tulbhaghia violacea* is one of the most harvested plants in the wild for its medicinal properties (Eloff, 1998).

The demand for this species is very high and supply cannot meet current and future demands, thereby warranting the utilisation of high-yielding plant cultivation technologies (Van Wyk et al., 2009). In this study, *T. violacea* plants were pH-stressed during hydroponic cultivation in an attempt to enhance its antifungal properties and these extracts were then tested against the fungus, *Fusarium oxysporum*. The compound allicin found in *T. violacea* is active against microbial infections caused by fungi, viruses, and bacteria in both plants and humans (Malungane, 2014). Studies that investigated the effects of different nutrient solutions and nutrient solution potential hydrogen (pH) on the growth and nutrient content of onions has been conducted, but not on *T. violacea* (Chad & Kane, 2003). Hence, this study aimed to assess the interactive effects of light and pH on plant growth, nutrient uptake and antifungal activity of *T. violacea* plants grown hydroponically.

4.2 Methods and materials

4.2.1 Plant materials

One-month old *T. violacea* plantlets were obtained from Best Western Seedlings Nursery (VarkensVlei Road, Phillip, Western Cape, 7785, South Africa) in six pack trays. The plantlets were then propagated using the division propagation method. The root clumps were divided and gently washed with tap water. The plantlets were then transplanted into 15 cm black plastic pots (Plastics for Africa, Somerset West, Cape Town, 7130) filled with river sand obtained from Builders Warehouse (Pty) Ltd, Cape Town. Plants were then placed on the concrete floor surface of the greenhouse and spaced 30 cm apart.

4.2.2 Greenhouse experiment

Experimental plants were grown under two light intensity conditions (low light or normal light) and were simultaneously treated to one of varying pH levels: pH 4, pH 6 and pH 8. The plants that were exposed to normal light received natural sunlight, which entered through the polycarbonate roof cover of the greenhouse, and the light intensity measured ranged from 300 lux to 500 lux. To obtain low light intensity, light transmission was reduced using black shading screen cloth (Alnet, Epping, Western Cape, South Africa). The cloth was hung four meters above the floor surface of the greenhouse and covered an area of 5 m^2. Plants were arranged in randomised blocks beneath the shade net. For each pH treatment, a group of twenty plants was randomly allocated to either low light intensity or normal light intensity. The greenhouse was covered with polycarbonate material and plants grown under full light were exposed to an 80% solar radiation transmittance while black shading screen cloth cast 40% shade on the plants. Plants were drip irrigated with Nutrifeed fertilizer (supplied by Starke Ayres, Cape Town). The fertilizer contained the following ingredients: N (65 mg/kg), P (27 mg/kg), K (130 mg/kg), Ca (70 mg/kg), Cu (20 mg/kg), Fe (1500 mg/kg), Mo (10 mg/kg), Mg (22 mg/kg), Mn (240 mg/kg), S (75 mg/kg), B (240 mg/kg) and Zn (240 mg/kg). The nutrient solution was prepared by dissolving 60 g of the fertilizer into a 60 L black reservoir filled with municipal tap water. An airstone was placed in each reservoir to add oxygen to the nutrient solution. About 250 ml of the suspension was applied to each plant using a handheld cylinder jar. To obtain the pH 4, pH 6 and pH 8 levels, the nutrient solution was adjusted using hydrochloric acid (HCl) to lower the pH or sodium hydroxide (NaOH) to raise the pH. The pH levels were monitored and maintained regularly using a JENCO vision plus instrument. The experiment was conducted at the nursery facilities of the Cape Peninsula University of Technology, Bellville, Western Cape, South Africa S33° 54' 0, E18° 38' 0 from February to April 2017 (2 months). The experiment was undertaken in a controlled environment greenhouse with the following temperature ranges: 25 ± 2 °C/17 ± 3 °C day/night and 74 ± 5% average relative humidity (RH).

4.2.3 Data collection

The heights of the plants were measured at weekly intervals for two months using a measuring tape. The measurements were recorded from river sand media level to the tip of the tallest shoot. Plants were harvested at the end of the experiment and fresh plant weights

were immediately captured. In order to determine the dry biomass, harvested plants were dried in the thermo-oven at 40 °C for 10-14 days.

4.2.4 Tissue analyses

Leaf samples were analysed for macro-and micro-nutrients by a commercial laboratory Bemlab (Pty) Ltd in Somerset West, South Africa. Leaves were washed with a teepols solution, rinsed with de-ionised water and dried at 70 $^{\circ}$C overnight in an oven. The dried leaves were then milled and ashed at 480 $^{\circ}$C and shaken up in a HCL (50%) solution for extraction through filter paper (Campell & Plank, 1989; Miller, 1998). The Potassium (K), Phosphorus (P), Calcium (Ca), Magnesium (Mg), Sodium (Na), Manganese (Mn), Iron (Fe), Copper (Cu), Zinc (Z), and Boron (B) content of the extracts were analysed using the Ash method. Total Nitrogen (N) content of the leaves was determined through total combustion in a Leco N-analyser. The amount of N, P, K, Ca and Mg were converted from percentage (%) to mg/kg, with 10 000 used as a conversion factor.

4.2.5 Solvent extraction

Plant materials (leaves and roots) from the different treatments were excised into smaller pieces and air dried at 35 ^{o}C. The dried materials from the different treatments were then ground separately to a fine powder using a Jankell and Kunkel model A 10 mill. The powdered bulbous root material (3 g) was extracted with 60 ml of acetone in a glass beaker and then filtered through with Whatman No.1 filter paper. Acetone is a useful extractant because it dissolves a wide range of hydrophilic and lipophilic compounds and is less toxic (Ellof, 1998). The extracted materials were then left to dry overnight using a fan, after which the dried acetone extracts were weighed to obtain the extract yield.

4.2.6 Minimum Inhibitory Concentration (MIC)

Fusarium oxysporum sp.glycines strain (UPFC no. 21) obtained through the courtesy of the Phytomedicine Programme, University of Pretoria, was used as the pathogenic agent. C. Cronje originally isolated the fungus strain from roots of a maize plant Delmas, Gauteng. The *F. oxysporum* was sub-cultured from stock agar plates and grown into Nutrient Broth (Merck, South Africa) for four hours. The fungal culture (100 ml) was added to each well of the 96-well microplates (10^5 cells/ ml). Amphotericin B (160 µg/ ml) was prepared as a stock

solution in acetone and served as a positive control while acetone was used as a negative control. Forty micro litre (40 µl) of 0.2 mg/ ml of *p*-iodonitrotetrazolium chloride (INT) (Sigma) was dissolved in sterile distilled water was added to each microplate well, sealed in a plastic bag and incubated at 37°C and 100% RH. The MIC values were recorded after 6, 12 and 18 h. The antifungal bioassay (MIC) consisted of three replicates per treatment and per watering interval.

4.2.7 Total Activity

The total activity is a very good criterion for comparing biological activities among plant species or cultivars because its formula takes into account the yield and antimicrobial activities of test extracts. The unit of TA is ml/g and it indicates the degree to which the active compounds in one (1) g of plant materials can be diluted and still inhibit the growth of the tested microorganisms (Eloff, 2000; Eloff, 2004).

4.2.8 Statistical analyses

The experimental data collected were analysed using one and two-way analyses of variance (ANOVA) (Scistatcalc, 2013) and Tukey HSD was used to separate the means at leaves of significance, $P<0.05$. These computations were performed using PAST software and graphs were plotted on MSExcel 2018.

4.3 Results

4.3.1 Height

The plant height ranged from 24-37 cm and did not vary significantly (df, 2, 57; $F=0.91$; $p > 0.05$) among pH treatments in both normal light and low light intensities for all the pH levels. However, plants grown under low light intensity produced the highest mean height values at pH 6 (37.35 ± 0.998 cm), closely followed by pH 4, and the lowest was obtained in pH 8 respectively (Figure 4.1). On the other hand, for normal light intensity, pH 8 treatment produced the highest mean height (29.35 ± 0.715 cm) followed by pH 6 and pH 4 (Figure 4.1). There was a significant difference in the heights of *T. violacea* between low and normal light intensities for all of the three-pH levels (Figure 4.1). Based on a two way Anova, the

interaction between light and pH was significant (df, 2; F=0.009; p < 0.001) in influencing growth of the studied species.

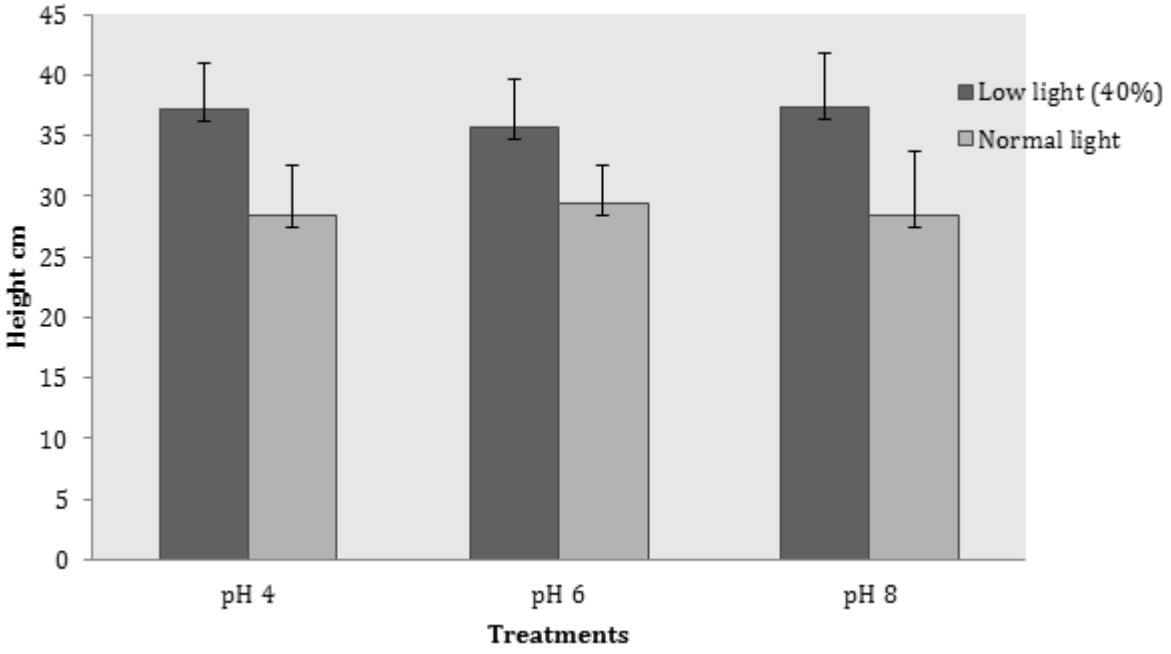

Figure 4.1: Mean ± SE heights of *T. violacea* grown under low light and normal light conditions while exposed to different pH at 2 months post treatment.

4.3.2 Number of leaves

There was no significant difference in the number of leaves for all treatments under normal light intensity (df, 2, 57; F=1.21; p < 0.3362) and low light intensity (df, 2, 57; F=1.21; p < 0.405) following one-way Anova analysis (Figure 4.2). The highest mean value of number of leaves was observed at pH 4 (10.9 ± 0.49 cm) compared to pH 6 (10.9 ± 0.331 cm) and pH 8 (10.55 ± 0.658 cm) under normal light, respectively. Surprisingly, the same mean value of the number of leaves obtained under normal greenhouse light intensity was also recorded under low light intensity for the same pH treatments. At pH 4, the highest number of leaves (11.65 ± 0.488 cm) obtained was comparable to pH 6 and pH 8 (10.55 ± 0.66 cm) under low light intensity (Figure 4.2). Based on a two way Anova, the interaction between light and pH was not significant (df, 2; F=1.09; p < 0.5) in influencing the number of leaves.

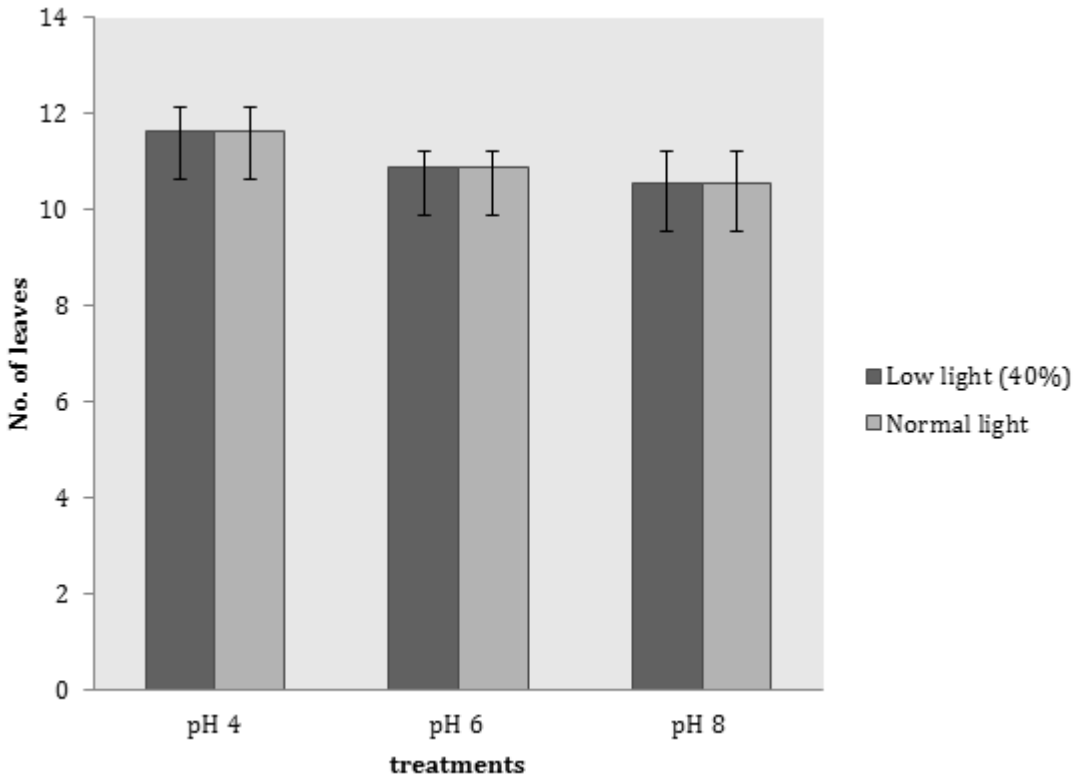

Figure 4. 2: Mean ± SE number of leaves of *T. violacea* grown under low light and normal light conditions while exposed to different pH at 2 months post treatment.

4.3.3 Fresh weight

There was no marked difference in plants' total weight among pH treatments under both normal light intensity (df, 2, 57; F=1.03; $p > 0.3293$) and low light intensity (df, 2,57; F=1.39; $p < 0.25$) conditions (Figure 4.3), even though plants grown under normal light intensity produced the highest mean value of total fresh weight in pH 4 (39.41 ± 3.77 g) followed by pH 6 and pH 8, compared to corresponding pH levels under low light intensity. However, for plants grown under low light intensity, high total fresh plant weight was recorded in pH 4 at 24.06 ± 2.105 g when compared to pH 6 and pH 8 was the lowest, respectively. Overall, the interaction between light and pH was significant (df, 2; F=0.001; $p < 0.001$) in influencing the fresh weight of this species.

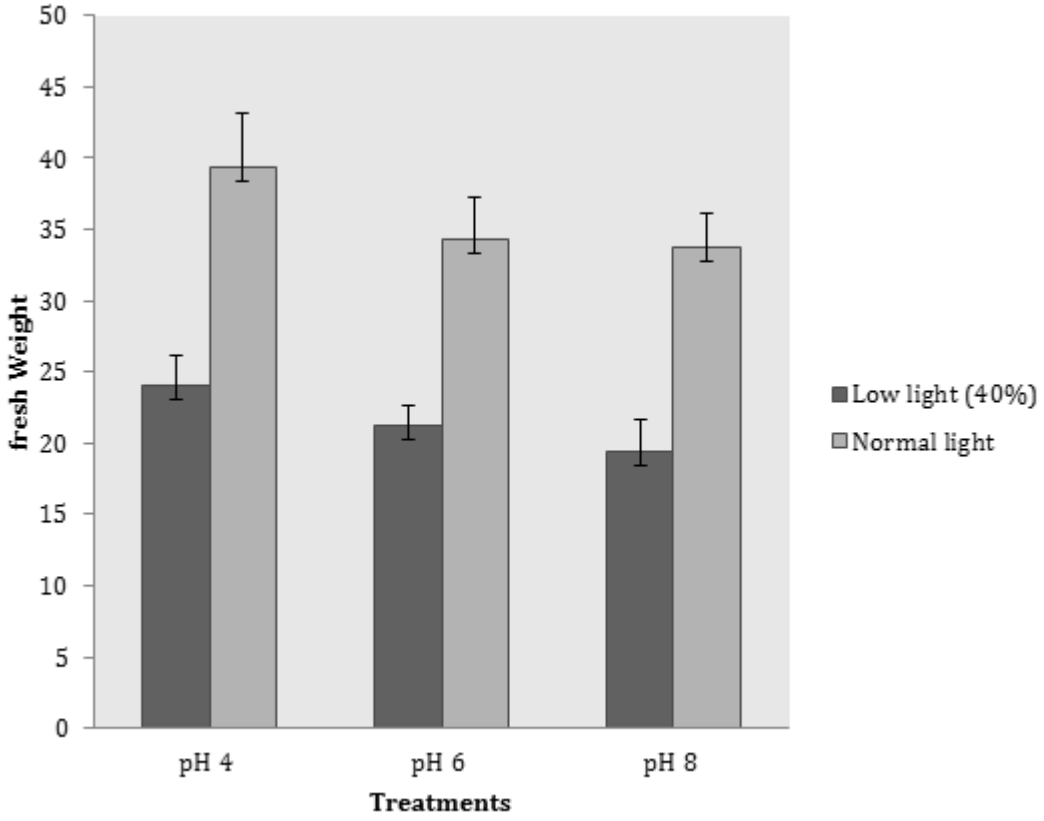

Figure 4.3: Mean ± SE fresh weights of *T. violacea* grown under low light and normal light conditions while exposed to different pH at 2 months post treatment.

4.3.4 Dry weight

The results revealed that there was a significant difference (df, 2, 57; F=12.63; $p < 0.05$) in total dry weights, under normal light intensity, pH 4 treatment (8.28 ± 0.802 g) produced the highest dry weight was compared to pH 6 and pH 8 treatments (Figure 4.4). On the other hand, under low light intensity, plant weights did not show significant variations ($p > 0.05$) among the different pH treatments (Figure 4.4); nevertheless, the highest mean value under low light was also obtained with plants at pH 4 (4.39 ± 0.392 g). Generally, normal light yielded a better mean dry weight than low light at lower pH levels. There was significant interactive effects (df, 2; F=6.4; $p < 0.05$) between pH and light intensity on the dry weight of this species.

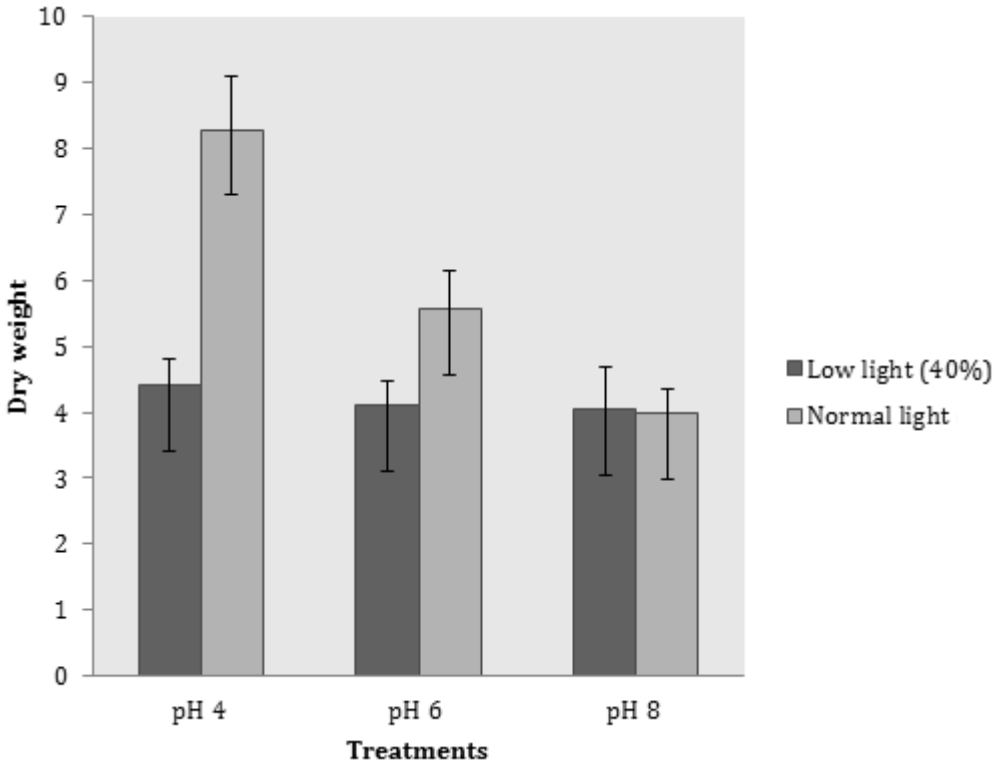

Figure 4.4: Mean ± SE dry weights of leaves of *T. violacea* grown under low light and normal light conditions while exposed to different pH at 2 months post treatment.

4.3.5 Tissue analysis

4.3.5.1 Macronutrient

Under normal light intensity, the level of macronutrient uptake (P, K, Ca, Mg, Na) in plants did not vary significantly ($p > 0.05$) among pH treatments (Table 4.1). The highest mean tissue nutrient contents in plants was obtained in plants exposed to pH 4 under normal light intensity where P value ranged from 67.5 ± 4.40 mg/kg, K (722.25 ± 9.31 mg/kg), Mn (100 ± 2.67 mg/kg) and Zn (80 ± 7.71 mg/kg). Plants at pH 6 under normal light intensity had the highest mean tissue value of Ca (138.75 ± 6.20 mg/kg) and B (74.25 ± 1.93 mg/kg) (Table 4.1). For plants exposed to both pH 8 and normal light intensity, the highest tissue nutrients levels were recorded for Mg (44.75 ± 1.18 mg/kg) and Na (7917 ± 241.10 mg/kg). For plants grown under low light intensity, the level of macronutrients (N, P, Ca, Mg, Na) were significantly different ($p < 0.05$) when pH levels were compared. The highest N uptake was recorded in plants exposed to pH 4 level under low light intensity (598.5 ± 5.693 mg/kg), P (59.5 ± 0.5 mg/kg), at pH 8 level plants macronutrients uptake for Ca (2025 ± 2.90 mg/kg), Mg (46.75 ± 3.79 mg/kg) and Na (7475.25 ± 306.05 mg/kg), respectively. The normal light

intensity showed the better results on the tissue P levels when compared to those under low light intensity for similar pH treatments.

Table 4.1: Tissue macronutrient contents (Mean ± SE) following exposure of *T. violacea* to different pH treatments and low and normal light intensity in the leaf at two months post-treatment.

Nutrient (mg/kg)	Treatments	Normal light	Low light (40%)
N	pH 4	546 ± 10.90A	598.5 ± 5.69A
	pH 6	577 ± 6.13A	447 ± 12.70B
	pH 8	571.75 ± 4.11A	561.5 ± 30.05 A
P	pH 4	67.5 ± 4.40B	59.5 ± 0.5A
	pH 6	55.75 ± 0.47A	38.5 ± 1.44B
	pH 8	52 ± 1.224A	50.25 ± 2.21A
K	pH 4	722.25 ± 9.31A	758.75 ± 10.30A
	pH 6	662.25 ± 8.61B	766 ± 11.45A
	pH 8	658.5 ± 5.79B	788 ± 8.41A
Ca	pH 4	127.75 ± 3.52A	138.25 ± 2.95A
	pH 6	138.75 ± 6.20A	202.5 ± 2.90B
	pH 8	106 ± 3.34B	130.5 ± 7.27A
Mg	pH 4	38.25 ± 0.75B	30.75 ± 0.85B
	pH 6	39.75 ± 1.88B	39.25 ± 2.86B
	pH 8	44.75 ± 1.18A	46.75 ± 3.79A
Na	pH 4	2090.5 ± 181.17B	1567.25 ± 99.75AB
	pH 6	1247.5 ± 63.21AB	2532.75 ± 247.88B
	pH 8	7917 ± 241.10A	7475.25 ± 306.05A

Means with the same uppercase letters in the same column are not significantly different and Means with same uppercase letters the same row are not significantly different ($p > 0.05$) following the Tukey test.

4.3.5.2 Micronutrient

For plants grown under normal light intensity, nutrient uptake of Mn, Zn, B was significantly different ($p < 0.05$) when pH treatments were compared (Table 4.2). The highest mean value of nutrient uptake on plants grown under normal light was obtained at pH 4 level for Zn (80 ± 9.714 mg/kg), Mn (100 ± 2.67 mg/kg) and, pH 6 for B (74.25 ± 1.931 mg/kg), respectively. The uptake of Cu and Fe in the pH treatments did not vary significantly in both low light and normal light intensity. In the low light intensity, there were significant differences ($p < 0.05$) among pH treatments for many tissue micronutrients. The highest Zn (60 ± 2.27 mg/kg) value on plants grown under low light intensity was recorded at pH 4 level, while at pH 8, Mn (581.5 ± 30.05 mg/kg) was the highest (Table 4.2). All pH treatments under both low and normal light intensity did not significantly affect the uptake of Iron. For Boron, pH treatments were significantly different when treatments were compared.

Table 4.2: Tissue micronutrient contents (Mean ± SE) following exposure of *T. violacea* to different pH treatments and low and normal light intensity in the leaf at two months post-treatment.

Nutrient (mg/kg)	Treatments	Normal light	Low light (40%)
Mn	pH 4	100 ± 2.67A	114.5 ± 2.21B
	pH 6	65.75 ± 5.07B	83.75 ± 3.42AB
	pH 8	17.75 ± 1.43AB	561.5 ± 30.05A
Fe	pH 4	232.25 ± 32.11A	116.75 ± 4.02B
	pH 6	168.25 ± 10.37B	137.5 ± 8.08B
	pH 8	202.25 ± 17.52A	165.25 ± 19.26 A
Cu	pH 4	3.25 ± 0.25A	4.75 ± 0.25A
	pH 6	1.75 ± 0.25B	2 ± 0.40B
	pH 8	1 ± 0 B	2 ± 0 B
Zn	pH 4	80 ± 9.71A	60 ± 2.27A
	pH 6	23.5 ± 1.32B	39 ± 1.22B
	pH 8	19.25 ± 1.70AB	36.5 ± 2.5B
B	pH 4	61.5 ± 4.11B	77.25 ± 2.17A
	pH 6	74.25 ± 1.93A	59.5 ± 1.84B
	pH 8	56.25 ± 1.65B	73.75 ± 0.94A
C	pH 4	37.625 ± 0.31A	37.63 ± 0.31A
	pH 6	38.6 ± 0.27A	38.6 ± 0.27A
	pH 8	38.72 ± 0.04A	38.72 ± 0.04A

Means with same uppercase letters in the same column are not significantly different and Means with same uppercase letters the same row are not significantly different ($p > 0.05$) following the Tukey test.

4.3.6 MIC

When corresponding pH treatments under low and normal light intensity were compared, there was no marked significant difference (df, 2, 6; F=6; $p > 0.05$). Generally, extracts from plants grown under low light intensity showed similar activity and MIC values ranged from 0.18 ± 0 to 0.375 ± 0.04 mg/ml at 6 h and 1.5 ± 0 to 0.97 ± 0.18 mg/ml at 18 h. The highest mean value of MIC was obtained when plants were subjected to a low light intensity. There was a significant difference ($p < 0.05$) between the pH 8 treatments under low and normal light intensity (Table 4.3). Acetone extracts of *T. violacea* treated at pH 8 level and exposed to normal light conditions, exhibited the strongest antifungal activity MIC value of 0.35 ± 0.04 mg/ml in the anti-*F. oxysporum* bioassay at 6 h-18 h, respectively (Figure 4.5). Therefore, based on the two way Anova, the interaction between light and pH was not significant in influencing antifungal extracts of this species.

Table 4. 3: Minimum inhibitory concentration (Mean ± SE) on *Fusarium oxysporum* by acetone extracts of *T. violacea* grown under low light or normal light conditions and simultaneously to one of varied pH at two moths post treatment.

Hours MIC	**Treatments**	**Normal light**	**Low light (40%)**
6hr	pH 4	0.18 ± 0B	0.28 ± 0.05A
	pH 6	0.33 ± 0.04A	0.28 ± 0.05A
	pH 8	0.37 ± 0A	0.117 ± 0.02B
12hr	pH 4	0.93 ± 0.09A	0.56 ±0.108A
	pH 6	0.656 ± 0.09B	0.56 ± 0.108A
	pH 8	0.93 ± 0.187A	0.47 ± 0.09B
18hr	pH 4	0.93 ± 0.187B	1.5 ± 0A
	pH 6	1.31 ± 0.18A	1.31 ± 0.18A
	pH 8	1.5 ± 0A	0.93 ± 0.18B

Means with same uppercase in the same row are not significantly different and Means with same uppercase in the same column are not significantly different ($p > 0.05$) following the Tukey test

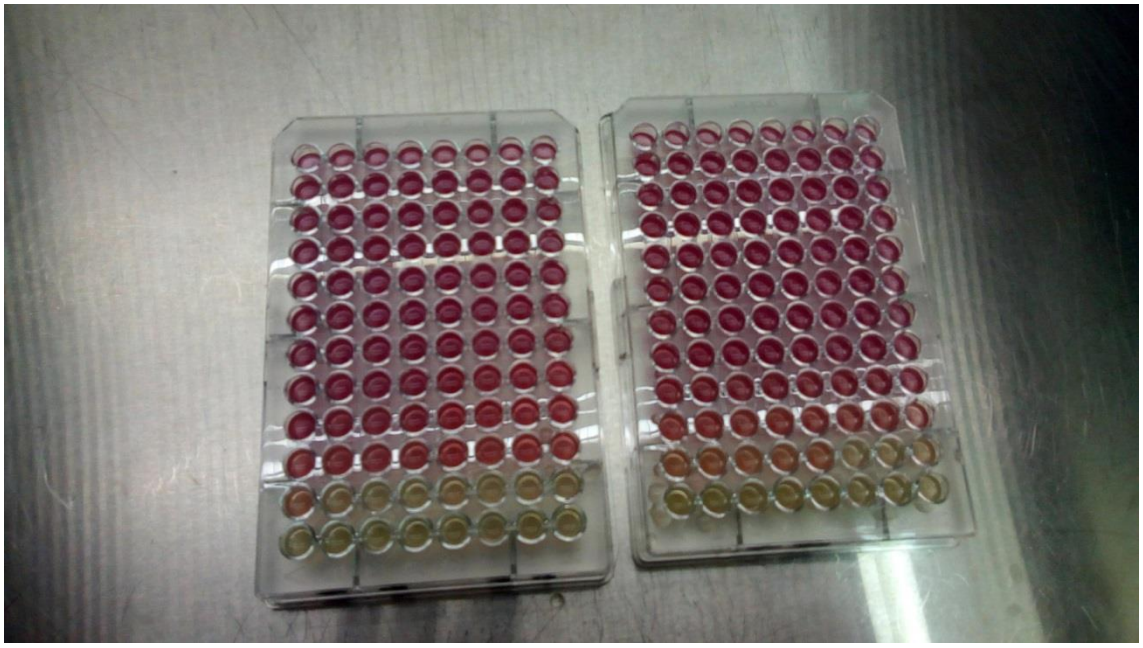

Figure 4.5: Anti-*Fusarium* activity of acetone extracts of *T. violacea;* photograph showing activity of the plant extracts (light red to light green colouration) in an MIC bioassay.

4.3.7 Total activity

Total activities reduce overtime from 6 h to 24 h for the various pH levels under low and normal light intensities. Among plants grown under normal light, there were significant differences in total activities obtained at the different pH levels with pH 8 consistently yielding the lowest total activities. Among plants grown under normal light at 6 h or 18 h, there was significant difference, while after 12 h, there was no marked difference when pH treatments were compared. The highest mean value of total activity was recorded at 6 h in pH 4 (47.55 ± 1.68 ml/mg) and at 18 h in pH 4 (10.33 ± 1.07 ml/mg) compared to pH 6 and pH 8 which was lowest at 18 h (3.44 ± 1.07 ml/g) in plants grown under normal light intensity (Table 4.3). Nonetheless, under low light intensity at 6 h, 12 hand 18 h, there was no significant difference among pH treatments. However, the highest mean value of total activity was obtained at 6 h in pH 6 (169.81 ± 42.09 ml/g) when compared to pH 8 (62.21 ± 13.42 ml/g), and pH 4 was the lowest (55.22 ± 12.46 ml/g). At 12 h and 18 h, plants grown under low light intensity pH 6 was the highest compared to pH 4 and pH 8. The highest mean value of total activity was obtained when plants were exposed to low light intensity even though there was no significant difference among the pH treatments. The interaction between light and pH on total activity was not significant (df, 2; $F = 6.4$; $p > 0.5$).

Table 4. 4: Total activity (Mean ± SE) of acetone extracts of *T. violacea* grown under low light or normal light conditions and simultaneously exposed to one of varied watering regimes at two moths post treatment.

Hours Total activity	Treatments	Normal light	Low light (40%)
6hr	pH 4	47.55 ± 1.68A	55.22 ± 12.469B
	pH 6	27.91 ± 4.38B	169.81 ± 42.096A
	pH 8	13.77 ± 4.302C	62.22 ± 13.422B
12hr	pH 4	10.33 ± 1.421B	36.1 ± 1.82AB
	pH 6	13.99 ± 2.22AB	42.5525 ± 10.56A
	pH 8	5.66 ± 1.941 C	31.1075 ± 6.70B
18hr	pH 4	10.33 ± 1.421B	18.1075 ± 0.91B
	pH 6	6.99 ± 1.110B	21.22 ± 5.23AB
	pH 8	3.44 ± 1.0C	13.55 ± 3.82B

Means with same uppercase in the same column are not significantly different and Means with same uppercase letters the same row are not significantly different following the Tukey test ($p > 0.05$).

4.5 Discussion

4.5.1 Growth

Plants grown under low light intensity at pH 4 obtained the highest mean value for height, compared to the other pH treatments. In a study done by Lefever (2013), pH 4 treated plants with supplementary phosphorus yielded the highest mean height, which was significantly greater when compared with other treatments of higher pH. It is well-known that nitrogen supplementation increases plant growth and biomass (Hipps et al., 2004). Plants treated at pH 4 and grown under low light intensity produced the highest mean value for number of leaves when compared with pH 6 and pH 8 treated plants. It was observed that the plants' fresh and dry weights decreased significantly as the pH increased. The highest mean dry weight was obtained in plants subjected to the pH 4 treatment and grown under normal light intensity,

while the lowest dry weight was obtained in plants subjected to pH 8. Hipps et al. (2004) also noticed a similar trend in their study. The findings of Anugoolprasert et al. (2012) is in agreement with this study, which showed that plants grown at pH 4.5 were heavier than plants grown at pH 5.7. In a similar experiment conducted by Aoestra (1968), vigorous growth for apple seedlings grown occurred at pH 3.8. This result may be because of a reduction in phosphorus, iron, and manganese since these nutrients modulate enzymes that are important for efficient photosynthesis. The highest nitrogen uptake was recorded in plants exposed to pH 4 under low light intensity. The availability of these nutrient elements plays a significant contributory role in the action of enzymes that stabilizes the metabolic processes that influence the plants' fresh weight (Stern, 2006; Brad and Weil, 2008). In order for carbohydrates to accumulate, it is highly influenced by temperature, relative humidity and light intensity, which directly affect photosynthetic efficiency in plants (Gomes-Laranjo et al., 2006). Therefore, based on the results obtained in this study, it is justifiable to argue that pH might have influenced nutrient uptake, which in turn influenced the growth of plants. This study further demonstrated that pH is an important determinant in how plants respond to light stress. Interactive effects were observed.

4.5.2 MIC and total activity

The MIC results obtained in this study indicate that the pH treatments have a greater significant influence on *T. violacea* roots extracts; more pronounced inhibition of growth of *F. oxysporum* was observed at pH 4 compared to pH 6 and pH 8 treatments after 18 h in the MIC bioassay. When plants were exposed to different environmental stresses, they accumulate high levels of bioactivity compounds (Ncube et al., 2011). Chad and Kane (2003) further emphasized that nutrient solution and pH significantly influenced onion plants' physiological variables. Previous studies done on *T. violacea* showed good MIC results when the extract where inhibited with the *Escherichia coli*, which is commonly found in HIV/AIDs patients (Ncube et al., 2013). The highest mean value of the total activity was obtained in plants subjected to pH 4 and grown under normal light intensity, while plants grown under low light intensity subjected to the pH 6 treatments were significantly higher than pH 4 and pH 8. In the tissue analysis, results obtained from the experimental plants for this study shows that nutrient elements such as K, Na, Mn, B and Cu were significantly enhanced in

plants subjected to pH 8 in both low light and normal light intensities. These nutrients also play a significant role in metabolic processes of the phenolic compound that may influence the medicinal properties of the plant (Xego, 2015). The accumulation of high levels of micronutrients could favour synthesis of secondary metabolites, corroborating the high total activities observed in a lower pH, especially among unshaded plants. *T. violacea* has been identified as a possible source of antifungal active compounds with the potential to be applied as a natural fungicide against plant pathogens in the agricultural industry (Eksteen et al., 2001).

4.6 Conclusion

This study has obtained the highest production of dry matter under a normal light conditions in plants subjected to pH 4, which also obtained the highest antifungal total activity of *T. violacea*. Therefore, for economic production, it could be ideal to cultivate this species at pH 4 under the natural light of a greenhouse in order to ensure high bioactivity of a natural fungicide. In conclusion, pH and light intensity interactively influenced the yield of *T. violacea* during cultivation. Extracts from plants exposed to both pH 4 and normal light intensity showed good activity against *F. oxysporum. T. violacea* can be manipulated for large commercial production.

CHAPTER FIVE

Effects of Light Intensities and Varying Watering Intervals on Growth, Nutrient Uptake and Antifungal Activities of Hydroponic Cultivated *Tulbaghia violacea* L under Greenhouse Conditions

Abstract

Optimization of the quality and quantity of medicinal materials during cultivation could improve the value of medicinal plants. This study was conducted to assess the interactive effects of pH and watering regime on plant growth, nutrient uptake and antifungal activity of *Tulbaghia violacea* plants grown hydroponically. *T. violacea* bulbs and leaves are used traditionally in Southern Africa for treatments of many ailments including gastrointestinal, asthma, fever, tuberculosis and cancer of the oesophagus. Experimental plants were grown under two light intensity conditions (low light or normal light) and were simultaneously exposed to one of three watering intervals: 5-day, 14-day and 21-day. Plant height, and plant fresh and dry weights were recorded. Significant ($p < 0.05$) variations in plant growths among the different watering regimes under normal light intensity were detected. Significantly, more macronutrients were detected in the tissues of plants at the higher frequency-watering interval. Plants grown under low light intensity yielded higher values for tissue micronutrients compared to normal light intensity. Interestingly, plants exposed to both extended watering interval period of 21 days and low light intensity had more bioactive crude extract against *F. oxysporum* in an MIC bioassay; 0.094 ± 0 mg/g compared to those shorter watering regime and higher light intensity. In conclusion, broadly, two trends occurred in the results — shorter watering interval and normal light intensity favoured plant growth and development, while plants grown under low light intensity with longer watering interval showed good bioactivity.

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5.1 Introduction

The most important ecological factor that influences the metabolic process of photosynthesis in plants is light (Humbert et al., 2007). When plants are exposed to low light intensity, they tend to have elongated leaves and reduced leaf surface area (Guo et al., 2012). Under conditions of high light intensity, plants produce high starch and carbohydrate contents, which contribute to their mass (Kose, 2014). However, when they are simultaneous exposed to more than one stress factors, the responses are not straightforward, for instance plants exposed to light and drought stresses invest more to produce shoots and leaves than roots and increase irradiation capture (Guo et al., 2012). Prider and Facelli (2004) argued that plants that are adapted to low light are sensitive to limited water supply, because a large proportion of biosynthesis meant for biomass is instead allocated to light-capturing organs, and this tend to create a larger transpiration area. Plants exposed to limited water and adequate light intensity may adjust to water stress in various ways. Plants may respond by reducing both leaf area and photosynthetic rate per unit leaf area through stomatal closure, decreases in chlorophyll fluorescence and chlorophyll or metabolic impairment through changes in photosynthetic carbon metabolism, such as accumulation of non-structural carbohydrates (Lawlor et al., 2002; Pinheiro and Chaves 2010; Basu et al. 2016). The interaction between shade and water stress can affect plant growth as observed by Liu et al. (2007) with *Abutilou theoprasti* L. Shading can reduce the impact of drought by limiting loss of water in soil during evaporation (Holmgren, 2000). The accumulation of such biosynthesised constituents in plants contributes to medicinal values that benefits human health (Lubbe & Verpole, 2011). Environmental factors play significant roles in the biosynthesis of secondary metabolite accumulation and enzymes activities. Therefore, understanding these environmental factors will help us to understand the dynamics of plant biomass, morphological changes and physiological mechanisms involved in plants immune responses (Caser et al., 2016). Fortunately, most exogenous factors like humidity, light, temperature and water are easily controlled in a greenhouse

While there are many publications on water deficient effects on the accumulation of secondary metabolites, only few reports have addressed the interaction of low light and water-deficit stresses. The study done by Charles et al. (1997) indicated that when plants (*Artimasia annuna* L.) are exposed to drought stress it produces higher secondary metabolite concentrations compared to well-watered plants. Factors such as land availability, water availability, season, climate, pests and diseases are major concerns during conventional

cultivation of indigenous plant species (Pierik, 1987; Arikat et al., 2004). The use of environmental-controlled greenhouses for plant production can be beneficial for the conservation of threatened plant species, such as *Tulbaghia violacea* (Alliaceae).

Medicinal plants are an important source and inspiration for discovery of new products for drug development (Xego et al., 2015). Hence, justifying why many research activities centre on the manipulation of these secondary metabolite constituents in plants in order to meet the demands of the pharmaceutical industry, traditional healers and the cosmetics industry (Bourgaud et al., 2001). In South Africa, *T. violacea* bulbs and leaves are traditionally used for treatments of gastrointestinal, ailments, asthma, fever, tuberculosis and the leaves are used to treat cancer of oesophagus (Kulkarni et al., 2005; Van Wyk et al., 2009). It has remained one of the most economically important species and is widely used in ethnomedicine, it is nutritive, and it is used as an ornamental plant. *T. violacea* is regularly harvested by traditional healers in the wild, a practice, which may cause decline of the species in the wild (Zschocke et al., 2000; Van Wyk et al., 2009; Jager and Van Staden, 2005; Mander and Mckenzie, 2005; Naidoo et al., 2008). Although *T. violacea* is still listed as 'Least Concern' on the 'National Red List of South Africa', it may eventually become threatened with extinction because of its high demand (Mander, 1999; Raimondo et al., 2009).

Many previous studies have validated the medicinal uses of the plant. Crude extracts from *T. violacea* showed good antimicrobial activities against bacterial strains (Ncube et al., 2011). *T violacea* has been proven to have similar anti-bacterial and antifungal activities as commercial garlic *Allium sativum* (garlic) (Brunetonn, 1995; Martindale, 1993). *Allium sativum*'s main pharmacological properties are bactericidal, virucidal, anti-fungicidal and anti-parisitic. Therefore, the objective of this study was to assess the interactive effects of pH and watering regime on plant growth, nutrient uptake and antifungal of extracts of *T. violacea* plants grown hydroponically.

5.2 Methods and materials

5.2.1 Plant materials

One-month old *T. violacea* plantlets obtained from Best Western Seedlings Nursery (VarkensVlei Road, Phillip, Western Cape, 7785, South Africa) in six pack trays. The

plantlets were then propagated using the division method. The root clumps were divided and gently washed with tap water. The plantlets were then transplanted into 15 cm black plastic pots (Plastic for Africa, Somerset West, Cape Town, 7130) filled with river sand supplied by Builders Warehouse (Pty) Ltd, Cape Town. Plants were then placed on the concrete floor surface of the greenhouse, spaced at 30 cm apart.

5.2.2 Experimental design

Experimental plants were grown under two light intensity conditions (low light or normal light) and were simultaneously treated to one of varying day watering intervals: 5-day, 14-day and 21-day. The plants that were exposed to normal light received natural sunlight that entered through the polycarbonate roof cover of the greenhouse, and the light intensity measured ranged from 300 lux to 500 lux. To obtain low light intensity, the light transmission was reduced using black shading screen cloth (Alnet, Epping, Western Cape, South Africa). The cloth was suspended four meters above the floor surface of the greenhouse and covered an area of 5 m^2. Plants were arranged in randomized blocks beneath the shade net. For each pH treatment, a group of twenty plants were randomly allocated to either low light intensity or normal light intensity. The greenhouse was made up of polycarbonate material and plants grown in full light solar radiance transmittance of 80% while the black shading screen cloth casted 40% shade. Plants were drip irrigated with Nutrifeed fertilizer (supplied by Starke Ayres, Cape Town). The fertilizer contained the following ingredients: N (65 mg/kg), P (27 mg/kg), K (130 mg/kg), Ca (70 mg/kg), Cu (20 mg/kg), Fe (1500 mg/kg), Mo (10 mg/kg), Mg (22 mg/kg), Mn (240 mg/kg), S (75 mg/kg), B (240 mg/kg) and Zn (240 mg/kg). The nutrient solution was prepared by dissolving 60 g of the fertilizer into a 60 L black reservoir filled with municipal tap water. An airstone was placed in each reservoir to add oxygen to the nutrient solution. About 250 ml of the suspension was applied to each plant. The experiment was conducted at the nursery facilities of the Cape Peninsula University of Technology, Bellville, Western Cape, South Africa from February to April 2017 (2 month). The research was conducted in a controlled environment inside a greenhouse, with a temperature that ranged from 24-26 °C during day and 15-20 °C during night. The average relative humidity was 74% RH.

5.2.3 Data collection

The height of the plant was measured at weekly intervals for two months using a measuring tape. The measurements were recorded from the river sand media level to the tip of the tallest shoot. Plants were harvested at the end of the experiment and fresh plant weights were immediately captured. In order to determine the dry biomass, harvested plants were dried in the thermo-oven at 40 °C for 7-14 days.

5.2.4 Tissue analyses

Leaf samples were analyzed for macro-and micro-nutrients by a commercial laboratory Bemlab (Pty) Ltd, Somerset West, South Africa. Leaves were washed with a teepols solution, rinsed with de-ionized water and dried at 70 °C overnight in an oven. The dried leaves were then milled and ashed at 480 °C shaken up in a 50:50 Hydrogen Chloride (HCL) (50%) solution for extraction through filter paper (Campell & Plank, 1989; Miller, 1998). The Potassium (K), Phosphorus (P), Calcium (Ca), Magnesium (Mg), Sodium (Na), Manganese (Mn), Iron (Fe), Copper (Cu), Zinc (Z) and Boron (B) content of the extracts were analysed using the Ash method. Total Nitrogen (N) content of the leaves was determined through total combustion in a Leco N-analyser. The amount of N, P, K, Ca and Mg were converted from percentage (%) to mg/kg.

5.2.5 Solvent extraction

Plant materials (leaves and bulbous roots) from the different treatments were excised into smaller pieces and air dried at 35 °C. The dried materials were then ground separately to fine powder using a Jankell and Kunkel model A 10 mill. The powdered root material (3 g) was extracted with 60 ml of acetone in glass beaker and was then filtered through with Whatman No.1 filter paper. Acetone is a useful extractant because it dissolves a wide range of hydrophilic and lipophilic compounds and is less toxic (Eloff, 1998). The extracted materials were then left to dry overnight using a fan and the dried acetone extracts were weighed to obtain extracted yield.

5.2.6 Minimum Inhibitory Concentration MIC

Fusarium oxysporum fungi sp. glycines strain (UPFC no. 21) was obtained through the courtesy of the Phytomedicine Programme, University of Pretoria and was used as the pathogenic agent. C. Cronje originally isolated the fungus strain from roots of a maize plant Delmas, Gauteng. The *F. oxysporum* strain was sub-cultured from stock agar plates and grown into Nutrient Broth (Merck, South Africa) for four hours. The fungal culture (100 ml) was added to each well of the 96-well microplates (10^5 cells/ ml). Amphotericin b (160 µg/ ml) was prepared as a stock solution in acetone and served as a positive control and acetone was used as a negative control. Forty micro litre (40 µl) of 0.2 mg/ ml of *p*-iodonitrotetrazolium chloride (INT) (Sigma) dissolved in sterile distilled water was added to each microplate well, sealed in a plastic bag and incubated at 37 °C and 100% RH. The MIC values were recorded after 6, 12 and 18 h. The antifungal bioassay (MIC) consisted of three replicates per treatment and per watering regime.

5.2.7 Total Activity

The Total Activity is a very good criterion for comparing biological activities among plant species or cultivars because its formula takes into account the yield and antimicrobial activities of test extracts. The unit of TA is ml/g and it indicates the degree to which the active compounds in one (1) g of plant materials can be diluted and still inhibit the growth of the tested microorganisms (Eloff, 2000; Eloff, 2004).

5.2.8 Statistically analysis

The experimental data collected were analysed using one and two-way analyses of variance (Anova) and Tukey HSD was used to separate the means at leaves of significance, $p < 0.05$. These computations were performed using PAST software and graphs were plotted on MS Excel 2018.

5.3 Results

5.3.1 Plant height

There was a significant difference (df, 2, 9; F=0.61; $p < 0.05$) in plant heights amongst the different watering regimes under normal light intensity. Plants subjected to the 5-day watering interval had a significantly higher mean value (25.15 ± 0.68 cm) when compared to those under 14-day watering regime (19.47 ± 0.63 cm) grown under normal light intensity (Figure 5.1). The lowest mean value for height was obtained in plants subjected to the 21-day watering interval under normal light intensity. In low light intensity, plant heights also varied significantly ($p < 0.001$) among the different watering regimes. The tallest plants occurred in 5-day watering regime (34.175 ± 0.863 cm) followed by the 14-day watering interval (28.77 ± 0.97 cm), and shortest was obtained in 21-day watering interval (23.47 ± 0.96 cm) under low light intensity see (Figure 5.1). When the same watering interval under low light and normal light conditions were compared to each other, higher mean values for height were obtained in plants grown under low light conditions for all three watering intervals. At the 14-day watering interval, plants grown under low light were significantly higher than those grown under normal light condition. The interaction between light and watering intervals was significant (df, 2; F=4.2; $p < 0.01$) in influencing the growth in height of this species.

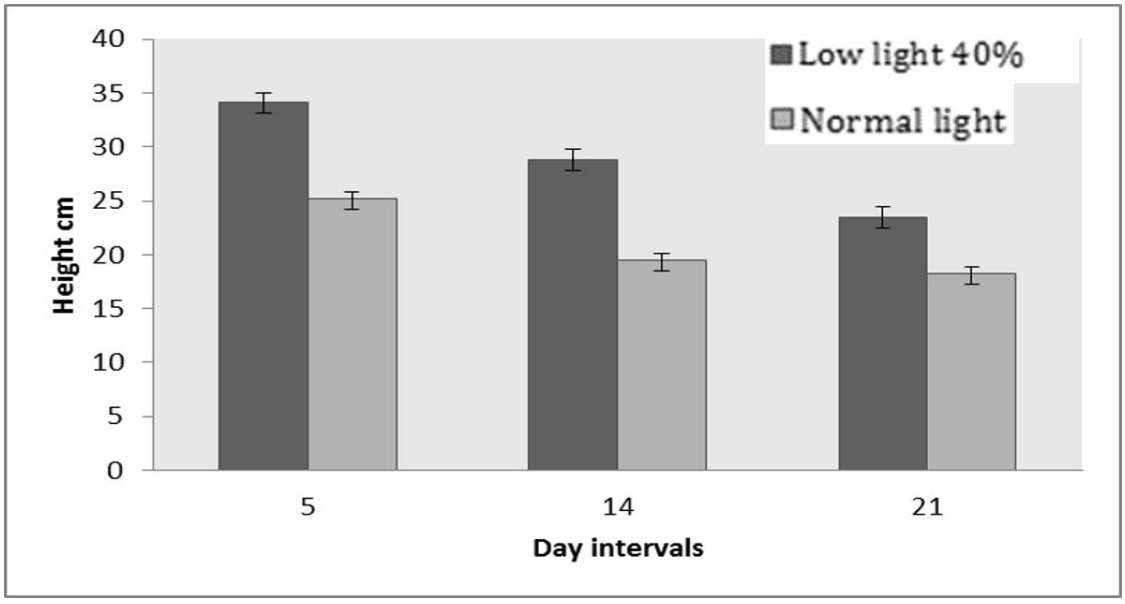

Figure 5.1: Mean ± SE heights of *T. violacea* grown under low light and normal light conditions while exposed to different watering regimes at 2 months post treatment.

5.3.2 Number of leaves

At normal greenhouse light intensity, there was a significant difference (df, 2, 9; F=0.84; $p < 0.05$) among watering intervals in the number of leaves produced (Figure 5.2). A high mean value for the number of leaves produced was recorded in plants subjected to 5-day watering intervals (10.3 ± 0.41) followed by the 14-day watering interval (10.25 ± 0.44) and the lowest was observed in 21-day watering interval (8 ± 0.37) grown under low light intensity. When 5-day and 14-day watering intervals were compared to each other in both low light and normal light conditions no significant difference ($p > 0.05$) was observed. However, 21-day watering interval under low light had significantly (df, 2, 9; F=0.23; $p < 0.05$) more leaves than the 21-day counterparts under normal light. No significant interaction between light and watering intervals (df, 2; F=4.2; $p > 0.05$) on number of leaves of this species was found.

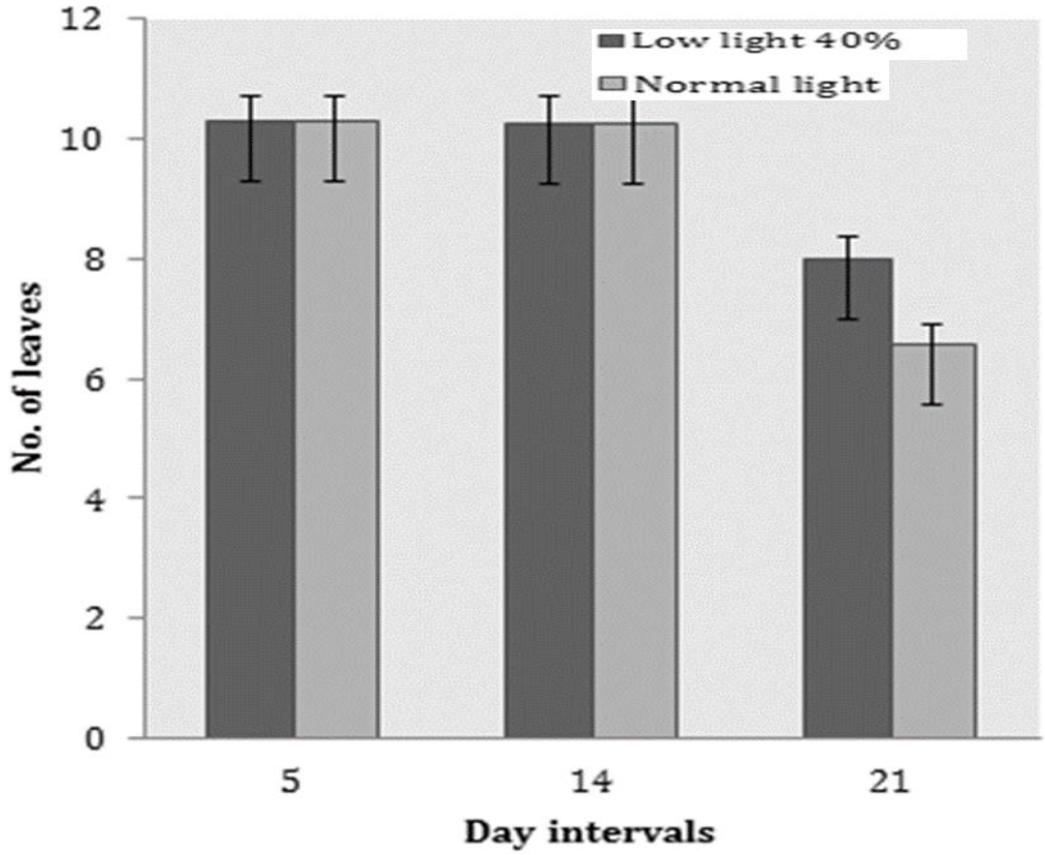

Figure 5.2: Mean ± SE number of leaves of *T. violacea* grown under low light and normal light conditions while exposed to different watering regimes at 2 months post treatment.

5.2.3 Fresh weight

There were significant differences (df, 2, 9; F=1.23: $p < 0.05$) among watering intervals in the mean fresh weights obtained after two months of cultivation under normal light intensity. Plants grown under normal light and watered every 5-day had significantly higher mean fresh weights (25.69 ± 2.11 g) when compared to both 14-day (16.89 ± 1.44 g) and 21-day (10.66 ± 0.8 g) watering regimes. The highest mean value for total fresh weight in plants grown under normal light intensity was recorded in the 5-day watering interval (25.69 ± 2.11 g) followed by the 14-day watering (16.89 ± 1.44 g) and lowest was obtained in the 21-day watering interval (10.66 ± 0.8 g) (Figure 5.3). In plants grown under low light intensity, there was a significant difference ($p < 0.05$) among the different day watering intervals. For low light, the highest mean value for total fresh weight was recorded in the 5-day watering interval, while the 14-day watering interval (14.56 ± 1.54 g) and 21-day day watering intervals (14.115 ± 1.161 g) followed (Figure 5.3). In this study, the highest mean values for total fresh weights were observed in plants grown under normal light intensity and watered at the 5-day interval. The interaction between light and watering intervals had a significant (df, 2; F=4.3; $p < 0.05$) influence on fresh weight of this species.

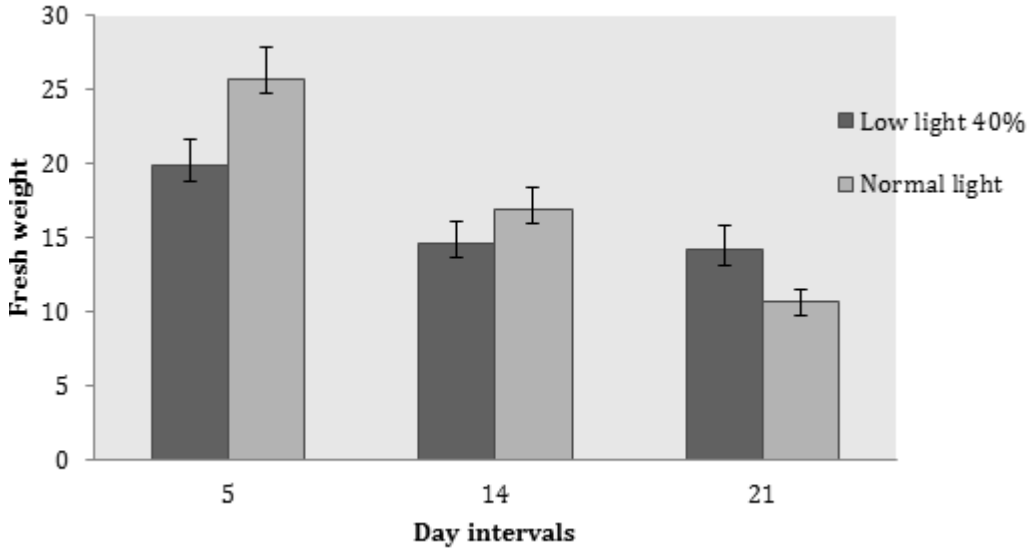

Figure 5.3: Mean ± SE fresh weights of *T. violacea* grown under low light and normal light conditions while exposed to different watering regimes at 2 months post treatment.

5.3.4 Dry weight

Under normal light conditions, the total dry weights significantly differed (df, 2, 9; F=1.53; p < 0.05) among the different watering regimes. The 5-day watering interval (4.96±0.51 g) treatment had the heaviest dry weight for leaves compared to the 14-day watering interval (3.34 ± 0.35 g) and 21-day watering interval (1.47 ± 0.16 g) for plants grown under normal light intensity (Figure 5.4). For plants grown under low light intensity, there was no significant difference obtained when day watering intervals (5, 14, and 21) were compared. Interestingly, plants grown under low light intensity while subjected to the 21-day watering interval was significantly higher compared to those subjected to the 21-day watering interval under normal light. Under high watering frequencies, plants exposed to higher light intensities performed better, yielding higher mean dry weights; however, the reverse was obtained at higher water-deficit conditions as mentioned above. Hence, unsurprisingly, a significant interaction was detected between light and watering intervals on the dry weight of *T. violacea* (df, 2, 9; F=7.3; p < 0.05).

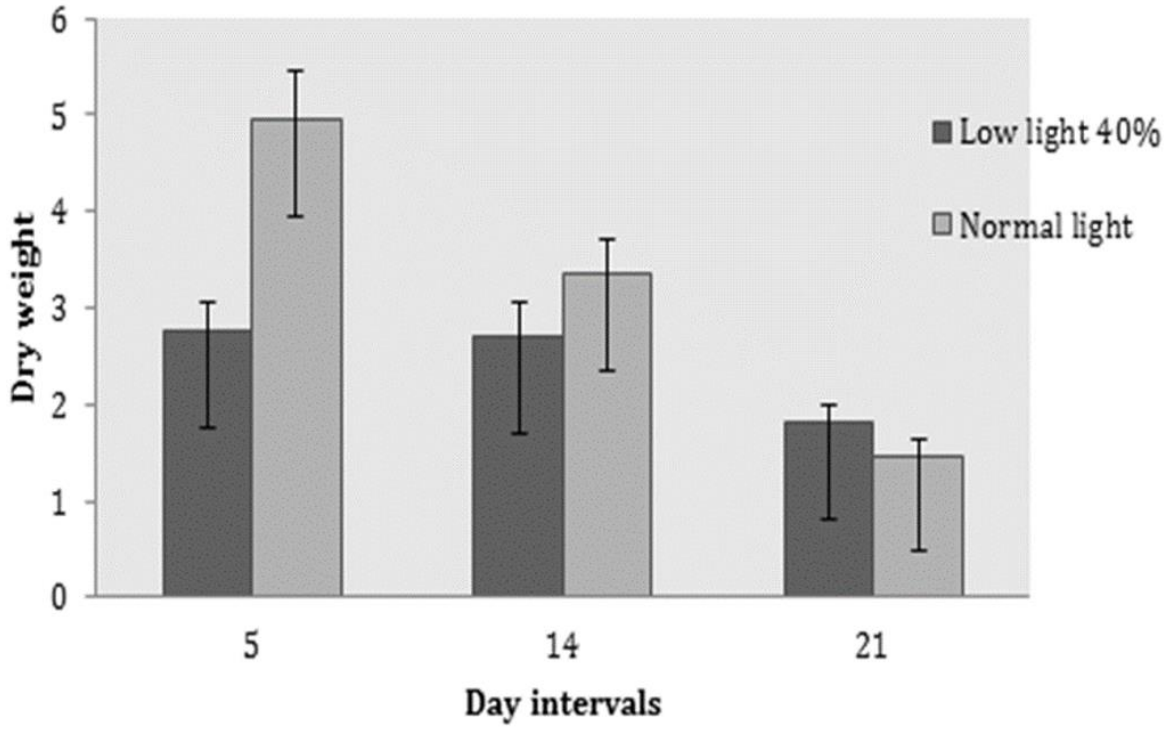

Figure 5.4: Mean ± SE dry weights of leaves of *T. violacea* grown under low light and normal light conditions while exposed to different watering regimes at 2 months post treatment.

5.3.5 Tissue Analysis

5.3.5.1 Macro-nutrients

Broadly, more macro-nutrients were taken up by plants in the higher frequency watering intervals. In this study, the level of N, P, K, Mg nutrient uptake differed significantly in plants ($p < 0.05$) among watering interval periods. However, there was no significant difference in plant tissue nutrient contents when watering intervals were compared ($p > 0.05$) for Na and Ca. Plant tissue nutrient contents of Na (2968.5 ± 165.55 mg/kg) and Ca (152 ± 11.20 mg/kg) obtained under normal light intensity and the 21-day watering interval were highest when compared to 5-day and 14-day watering intervals (Table 5.1). Macronutrients (N, P, K, Ca, Mg and Na) in the tissue of leaves of plants grown under low light intensity varied significantly ($p < 0.05$) among the different watering intervals. The uptake of N (607.75 ± 4.02 mg/kg), P (57.5 ± 1.04 mg/kg) and K (802.25 ± 3.25 mg/kg) increased significantly in leaves under the 5-day watering regime, while Mg (45.25 ± 2.35 mg/kg), Ca (209.5 ± 7.23 mg/kg) and Na (6048 ± 183.36 mg/kg) levels were higher in 14-day watering regimes grown under low light intensity.

Table 5.1: Tissue macronutrient contents (Mean ± SE) for *T. violacea* grown under low light and normal light conditions while being exposed to one of varied watering regimes at two moths post treatment

Macro-nutrients	Day interval	Normal light	Low light (40%)
N	5	559.25 ± 9.67A	607.75 ± 4.028A
	14	488.75 ± 36.545B	391 ± 3.785AB
	21	477.25 ± 11.345B	463.25 ± 12.512B
P	5	50.25 ± 1.652A	57.5 ± 1.04A
	14	30 ± 5.461B	25.5 ± 1.89C
	21	39.75 ± 1.652B	35.5 ± 1.44B
K	5	685.5 ± 4.627A	802.25 ± 3.25A
	14	450.75 ± 82.799B	703 ± 24.09B
	21	565.5 ± 10.267AB	653.25 ± 19.71AB
Ca	5	146.75 ± 12.598A	136 ± 3.488B
	14	143.75 ± 36.051A	209.5 ± 7.23A
	21	152 ± 11.202A	167.5 ± 5.95B
Mg	5	55.5 ± 3.796A	40 ± 0.577A
	14	34.25 ± 8.107B	45.2 ± 2.35A
	21	38.75 ± 2.286B	34.5 ± 1.190B
Na	5	2917.5 ± 126.30A	2654.5 ± 62.430C
	14	2926.75 ± 645.526A	6048 ± 183.366A
	21	2968.5 ± 165.55A	4810 ± 91.357B

Means with same uppercase letters in the same column are not significantly different and Means with same uppercase letters the same row are not significantly different following the Tukey test ($p > 0.05$).

5.3.5.2 Micro-nutrients

Generally, plants grown under the low light intensity revealed higher values for tissue micro-nutrients. Plants under normal light intensity, the tissue nutrient contents of Mn, B and Zn in day watering intervals increased significantly ($p < 0.05$) in the leaves of *T. violacea*. Nevertheless, the Fe and Cu content did not differ significantly among the different watering regimes. Tissue nutrient content of B (91.5 ± 4.62 mg/kg), Mn (59 ± 2.48 mg/kg) and Zn (29.75 ± 1.43 mg/kg) increased significantly in 5-day watering regime under normal light intensity, while C (3825 ± 32.27 mg/kg) levels were higher in 21-day watering regime plants grown under normal light intensity (Table 5.2). The nutrient content of Zn, Cu, and Fe under low light intensity did not show significant differences among the watering regimes. However, there was a significant difference on the tissue nutrient content of Mn and B under low light condition. The nutrient content for B (85.75 ± 1.10 mg/kg) and Mn (61.5 ± 2.25 mg/kg) increased significantly in plants grown under low light intensity and subjected to the 5-day watering interval when compared to 14-day and 21-day watering intervals.

Table 5.2: Tissue micronutrient contents (Mean ± SE) for *T. violacea* grown under low light and normal light conditions and simultaneously exposed to one of varied watering regimes at two moths post treatment

Micronutrient (mg/kg)	**Watering intervals (day)**	**Normal light**	**Low light (40%)**
Mn	5	59 ± 2.483A	61.5 ± 2.25A
	14	26.5 ± 5.80B	30 ± 2.67B
	21	27.25 ± 2.015B	15.5 ± 0.866B
Fe	5	196.25 ± 21.70A	113.75 ± 7.68A
	14	129.75 ± 33.66A	176.5 ± 14.121A
	21	121.75 ± 10.28A	115 ± 12.82A
Cu	5	2.75 ± 0.25A	2.75 ± 0.47A
	14	2.5 ± 0.64A	2 ± 0A
	21	2 ± 0A	2.5 ± 0.64A
Zn	5	29.75 ± 1.43A	31 ± 2.12B
	14	21.25 ± 3.70A	40.75 ± 3.32A
	21	23.5 ± 0.64A	31.5 ± 1.32B
B	5	91.5 ± 4.62A	85.75 ± 1.108A
	14	39.5 ± 9.18B	61.25 ± 2.95B
	21	46.75 ± 1.65B	42.25 ± 1.314C
C	5	3782.5 ± 75.97A	3782.5 ± 75.97A
	14	3675 ± 62.51A	3675 ± 62.51A
	21	3825 ± 32.27A	3825 ± 32.27A

Means with same uppercase letters in the same column are not significantly different and Means with same uppercase letters in the same row are not significantly different following the Tukey test ($p > 0.05$).

5.3.6 MIC

There was no significant different ($p > 0.05$) between the different watering interval treatments. Based on the MIC results, the strongest inhibition against anti-*F. oxysporum* was observed in plants subjected to 5-day watering regime under normal light with the value of (0.094±0 mg/ml) after 6 h followed by subjected to 14-day watering regime. Nevertheless, the interaction between light and watering interval in influencing the antifungal activity of this species was not significant (df, 2; $F=1.1$; $p > 0.5$) (Table 5.3).

Table 3: Minimum inhibitory concentration (Mean ± SE) on *Fusarium oxysporum* by acetone extracts of *T. violacea* grown under low light or normal light conditions and simultaneously to one of varied watering regimes at two moths post treatment

Hours	**Watering intervals (day)**	**Normal light**	**Low light 40%**
6hr	5	0.094 ± 0AB	0.094 ± 0AB
	14	0.281 ± 0.054B	0.281 ± 0.054B
	21	0.375 ± 0A	0.375 ± 0A
12hr	5	0.375 ± 0B	0.375 ± 0B
	14	0.375 ± 0B	0.375 ± 0B
	21	0.75 ± 0A	0.75 ± 0A
18hr	5	0.75 ± 0B	0.75 ± 0B
	14	0.75 ± 0B	0.75 ± 0B
	21	1.5 ± 0A	1.5 ± 0A

Means with same uppercase letters or letters are in the same column are not significantly different and Means with same uppercase letters the same row are not significantly different following the Tukey test ($p > 0.05$).

5.3.7 Total activity

The highest Total activity value for was recorded in the roots of *T. violacea* plants that was cultivated under normal light intensity, subjected to the 5-day watering interval and at an exposure of 6 hours (85.10 ± 5.22 ml/g) and at 12 hours (85.10 ± 5.22 ml/g) (Table 5.4). The second highest mean value was recorded at 18 hours (11.77 ± 1.28 ml/g), at the 14-day watering interval while the lowest mean value was recorded at the 21-day watering intervals. Nevertheless, plants' roots extracts grown under low light intensity showed significant difference against *F. oxysporium* among day watering intervals. The highest total activity extract of *T. violacea* cultivated under low light intensity against *F. oxysporum* was recorded

in the 21-day watering interval (118.39 ± 3.83 ml/g) at 6 hours compared to the 14-day watering interval (78.80 ± 12.64 ml/g) while the 5-day watering interval obtained the lowest mean value (37.10 ± 1.17 ml/g) (Table 5.4). In the present study, plants roots extracts against *F. oxysporium* grown under normal and low light intensity shows good total activity; however, the highest mean values were recorded in plants roots extracts grown under low light intensity conditions subjected to the day 21 watering interval. In this case, the interaction between light and watering interval was significant (df, 2; F=56.1; $p < 0.01$) in influencing total activity of the extracts of this species.

Table 5.4: Total activity (Mean ± SE) of acetone extracts of *T. violacea* grown under low light or normal light conditions and simultaneously exposed to one of varied watering regimes at two moths post treatment

Hours	**Watering intervals (day)**	**Normal light**	**Low light (40%)**
6hr	5	85.1025 ± 5.220A	37.1075 ± 1.170C
	14	41.73 ± 7.5865 B	78.8025 ± 12.649B
	21	20.8825 ± 4.955C	118.395 ± 3.834A
12hr	5	85.1025 ± 5.220A	18.55 ± 0.584C
	14	10.44 ± 1.908B	39.995 ± 7.596 B
	21	7.3275 ± 3.243C	29.33 ± 0.629A
18hr	5	11.775 ± 1.289A	9.2575 ± 0.265B
	14	8.44 ± 0.479B	13.6075 ± 1.020B
	21	5.2175 ± 1.239C	14.6625 ± 0.313B

Means with same uppercase letters in the same column are not significantly different and Means with same uppercase letters the same row are not significantly different following the Tukey test ($p > 0.05$).

5.4 Discussion

5.4.1 Plant growth

The plants grown under low light conditions produced high mean values for height than those grown under normal light conditions. Research done by Sack et al. (2003) reported that shade can benefit water stress by decreasing overheating, reduce transpiration and oxidative stress. Plants respond to these conditions by accumulating carbohydrates in leaves, and these soluble sugars contributes to turgor maintenance and limit water losses (Morgan, 1984; Abrams, 1986; Auge et al., 1998). These arguments plausibly explain why taller plants were observed in low light conditions than in normal light intensity in this study. The number of leaves produced did not statistically differ in both low light and normal light conditions even though plants that simultaneously grew under low light and 21-day watering regimes showed a significant difference in leaf numbers when compared with those grown under normal light intensity under the same watering regime. The study done by Lof et al. (2005) on seedling response of *Quercus* species indicated that limited light availability overrules the impact of limited water, and they postulated that under these conditions, plants will allocate more carbohydrates to growth and non-photosynthetic organs.

In the current study, plants grown under normal light while subjected to the 5-day watering interval obtained the highest mean value for total fresh weight when compared to 14-day and 21-day watering intervals. These results are consistent with the findings of Xego et al. (2015) which indicate that *Siphonochilus aethiopicus* produced more weight after growth when this species was irrigated at shorter watering intervals (5-day). However, plants grown under low light conditions had a significant decrease in their total fresh weight when compared to those grown under normal light conditions.

5.4.2 Tissue analysis

Broadly, two trends occurred in the results, firstly higher concentrations of macro-nutrients were absorbed by plants in the higher frequency watering intervals with, and secondly, plants grown under low light intensity revealed higher values for tissue macro-nutrients. Plants grown under the low light conditions were statistically different in the absorption of macronutrients for plant growth. Hence, the height of *T. violacea* plants under low light was higher than those grown under normal light conditions. Macronutrients have a strong influence on plant growth in the presence of water, and when plants are exposed to shade conditions the transpiration rate from the soil is reduced, overruling the negative effects of water-stress (Prider and Facelli, 2005). Cuervo et al. (2012) suggested that due to changes in physical, chemical and microbiological properties micronutrients are difficult to track. In the current study, plants that were watered at the 21-day interval had the lowest tissue nutrient content, agreeing with Signh (2009), which reported that decreases in watering levels can reduce the availability of nutrient uptake and their transportation (Jeminez et al., 1996).

5.4.3 MIC and Total activity

There are few studies done on the effects of low light intensity and limited water on the photochemistry of plants (Kitao et al., 2000). In plants subjected to both the 21-day watering interval, low light intensity produced more leaves than those grown under normal light intensity. Yang (2008) argued that shade aggravates the drought stress and plants in the shade invest more to produce shoots and leaves than biomass. According to Eloff (2016), limited water supply lead to water stress in plants. In this study the mean weight of the 21-day watering interval plants were significantly lower when compared to the 5-day and 14-day watering intervals at normal light intensity.

Plants exposed to drought stress produces higher concentration of secondary metabolites than those that are well watered when cultivated (Selmar & Kleinwacther, 2013; Alinian et al., 2016). In this study, plants watered after 21-day produced a high total activity in the *T. violacea* root extract against *F. oxysporum* under low light intensity compared to those under normal light intensity. This clearly indicates that there was an interaction between watering regimes and light intensity. These results correspond with findings of Charles et al. (1993) where *A. annua* plants that were exposed to long-term drought had increased concentrations of the artemisinin compound, known for its beneficial effects on human health. The best results for antifungal inhibitory effects against microorganism were obtained in the day 21 watering interval under low light conditions.

5.5 Conclusion

In conclusion, the total weight of aerial parts of *T. violacea* increased with shorter watering frequencies and increased antifungal activity was associated with prolonged watering intervals. These results suggested that nutrient absorption and subsequent tissue nutrient levels might be modulating the responses, such as plant biomass and antifungal activity in relation to light intensity and watering regime. Finally, the findings of this study will benefit the cultivation of this species and contribute toward achieving enhanced yield of bioactive materials from medicinal plants. Wild *T. violacea* can be conserved, secondary metabolites can be manipulated and consistent supply of this plant guaranteed through large scale commercial cultivation.

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