The Effects of Varying pH and Shading on Antioxidant Content-and -capacity of *Tulbaghia violacea*, Cultivated Hydroponically under Greenhouse Conditions

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Abstract— Environmental stress factors such as high or low soil pH, water deficit, high temperature, and shade may induce accumulation of reactive oxygen species in plants, which may lead to oxidative stress when in excess. The effects of varying the environmental variables such as pH and light intensity on secondary metabolite contents and the antioxidant capacity of extracts of *T. violacea* under greenhouse conditions, with the aim of improving the hydroponic cultivation of *T. violacea*, were assessed. In this study, six weeks old seedlings of *T. violacea* were separately exposed to two levels of light intensity and two levels of pH (4 and 8) in a greenhouse. The total polyphenol, alkaloid, and flavonol contents and antioxidant activities in the leaf and bulbous root extracts were determined using spectrophotometric methods. The total polyphenol contents of the leaves were significantly higher ($df = 1, 4$; $P < 0.05$) in plants under 40% shading and pH 8 compared with 0% shading and pH 4, respectively. Although generally, higher total polyphenol and flavonol contents occurred in the roots than in the leaves, the roots had a significantly ($P < 0.05$) reduced flavonol content compared with the leaves of plants in the 40% shade treatment. The antioxidant activity was more pronounced in the leaf extract from plants maintained under 40% shade or pH 8 than 0% shade or pH 4, respectively. In conclusion, 40% shading and high pH (pH 8) correlated with a high accumulation of antioxidants and antioxidant activities in *T. violacea*.

***Keywords*—** Antioxidant activities, *Tulbaghia violacea*, pH, light intensity, antioxidant contents

I. Introduction

Environmental stresses, such as too high or too low temperature, pH, light, and water levels can cause oxidative stress in plants, which is mediated by free radicals/reactive oxygen species (ROS) generation. Oxidative stress is a state of imbalance between the production of ROS and the neutralization of free radicals by antioxidants. It is associated with the damage of cellular components, including lipids, nucleic acids, metabolites, and proteins, subsequently leading to the death of plant cells [1].

In response to numerous stresses, plants have evolved adaptations that enable them to mitigate the negative effects of environmental stresses. For example, plants produce more secondary metabolites polyphenols, and alkaloids [2]. Numerous plants and their secondary metabolites have been reported to possess antioxidant properties [3], [4]. Antioxidants found in plant extracts can prevent free radicals from damaging tissue or organs [5]. According to [6], oxidation is defined 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 [7]. Antioxidant compounds produced by plants during stress conditions can be used against free radicals that predispose humans to sickness and diseases [8]. Therefore, there is a huge interest in manipulating medicinal plants to produce higher quantities of medicinal materials that are bioactive against pathogens and free radicals through innovative cultivation protocols.

High-tech plant cultivation methods such as tissue culture and hydroponics offer many opportunities to manipulate plant cultivation to achieve high-quality production, optimize yield and achieve uniformity [9]. 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 [10]. It was indicated by [9] that growing plants in a controlled environmental condition could lead to the manipulation of phenotypic variation of the concentrations of biological compounds being produced.

Tulbaghia violacea is a drought-resistant species, and it occurs in South Africa (Eastern Cape, KwaZulu-Natal, and

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Limpopo provinces) and Zimbabwe. This fast-growing bulbous plant is a potential source of antioxidants. While many studies have shown that crude extracts of *T. violacea* are bioactive against the nematode, fungal and bacterial pathogens [11]-[13], 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 be a rich source of micronutrients, such as Vitamin C, Vitamin E, Boron, and beta-carotene, that contribute to the scavenging and reduction of free radicals in diets [14], [15]. Although *T. violacea* is classified as 'Least concern' in the Red list database in South Africa, the need to optimize its desirable pharmacological properties warrants the search for optimum cultivation practices for this species [16]. 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.

II. Materials And Methods

A. Plant material

One-month-old *T. violacea* seedlings (Silverlace cultivar) supplied by Best Western Seedlings Nursery (VarkensVlei Road, Philippi, 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 (Plastics for Africa PTY/LTD, Somerset West, Cape Town, 7130) filled with sterile river sand from Builders Warehouse (Pty) Ltd, Cape Town.

B. Greenhouse experimental design

To evaluate the effects of exogenous environmental factors on plant growth, secondary metabolite contents, and antioxidant activities, the seedlings of *T. violacea* were exposed to two stress factors with different treatment levels: two levels of light intensity (low and high light), three levels of pH (4, 6, and 8). The plants were randomly allocated to each treatment and in a completely randomized design. For light intensity treatments, experimental plants were cultivated under one of two shade levels (0 and 40% shade). The 0% shade treatment was achieved by exposing plants to natural sunlight that entered through the greenhouse's roof, while the 40% shade was achieved by covering the plants in the same greenhouse with a 40% shade net (Allnet, Epping Industria). During the study period, from January to February, the average light intensity range was 300-500 lux during the day. The pH levels (4. 6, and 8) 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 using the drip irrigation system. The 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 effects of light intensity and pH on antioxidant content and -capacity were assessed on the tested plants.

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 a maintained temperature between 24–26 °C during the day and 15–20 °C at night. The average humidity was 74%.

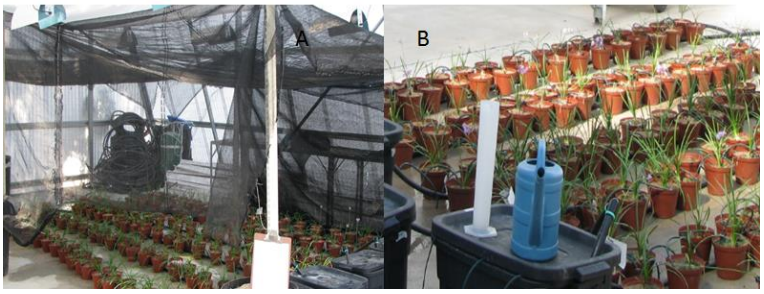

Fig. 1: Setup of the exposure of *T. violacea* under A) low light intensity B) high light intensity

C. The antioxidant analysis

For the antioxidant analysis, harvested materials were air-dried at 35 ^{o} C for 7–14 days. The dried plants were then separated into leaves and bulbs and ground into a fine powder using a Junkel and Kunkel model A 10 mill. The ground powder was then stored in air-tight stopper glassware before analyses. To obtain a crude extract, the finely ground leaf and bulb materials of this plant were then stirred separately in Ethanol (EtOH) (Saarchem, South Africa), and thereafter centrifuged at 4000 rpm for 5 min [17]. The crude extract of this species was used for the below-mentioned chemical analyses.

Determination of antioxidant capacity (FRAP)

FRAP assay was performed using the method of [18] 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).

Trolox Equivalent Antioxidant Capacity (TEAC)

The TEAC method was employed to measure the free radical scavenging ability of the antioxidants in *T. violacea* plants as described by [20]. The TEAC value is based on the antioxidant's ability to scavenge 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) radical ($ABTS^{\bullet+}$) radical cation, which has a blue-green color, relative to the $ABTS^{\bullet+}$ radical cation scavenging ability of the water-soluble vitamin E analog.

Determination of polyphenol, flavonol, and flavanone contents

Polyphenol: The Folin Ciocalteu method was used to determine the total polyphenol content of the various crude extracts [21], [22]. Twenty-five microliter of the sample was mixed with 125 µL Folin–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 were 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).

Flavonol: 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 extract 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 [23]. The results were expressed as mg quercetin equivalent per g dry weight (mg QE/g DW).

D.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.

III. Results

Light intensity influenced secondary metabolite content in the leaves and bulbous roots of *T. violacea*. The polyphenol contents of the leaves ranged from 2.9 to 5.8 GAE/g DW (Fig. 2) and were significantly higher ($df = 3,8$; $F = 5.3$; $P < 0.05$) in 40% shaded plants (high light-exposed plants) than in the 0% shaded-plants (higher light intensity).

The polyphenol content of *T. violacea* in the leaves was significantly ($df = 1, 4$; $P < 0.01$) increased at the higher pH (8.0) (4.75 mg GAE/g dry weight) compared to the lower pH 4 treatment (2.64 GAE/g) (Fig 2). Generally, polyphenol content was significantly higher in the roots than in the leaves in both pH and light intensity treatments.

The flavonol content in the leaves and roots did not vary significantly ($df = 1, 4$; $P > 0.05$) when pH 4 and pH 8 were compared (Fig. 2). The roots had a significantly ($df = 1, 4$; $P < 0.05$) reduced flavonol content compared to the leaves of plants grown under 40% shade.

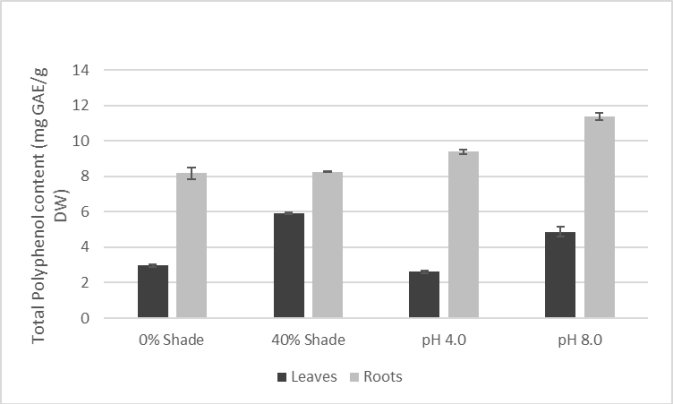

Fig. 2: The total polyphenol (mg GAE/g dry weight) content of the leaves and roots of *T. violacea* plants under different light intensities. Values represent the means ± SD for the leaves (A) and roots (B) (n = 18).

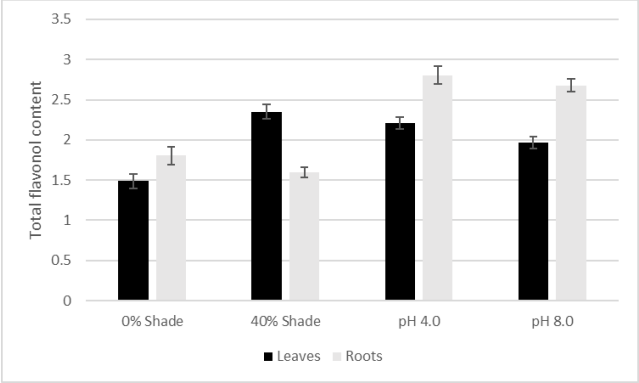

Fig. 3: The total flavonol (mg GAE/g dry weight) content of the leaves and roots of *T. violacea* plants under different light intensities. Values represent the means ± SD for the leaves (A) and roots (B) (n = 18).

Antioxidant capacity

Leaves of plants that were exposed to the higher pH yielded higher antioxidant activities in FRAP and TEAC when compared to lower light intensity for the aerial part (df = 3, 8; $P < 0.001$) (Fig. 4). However, the FRAP value was slightly lower in the 0% shaded plants than the 40% shaded plants for the bulbous roots and aerial materials; however, the difference was not significant ($P > 0.05$). Overall, the roots had a higher antioxidant activity based on the FRAP results.

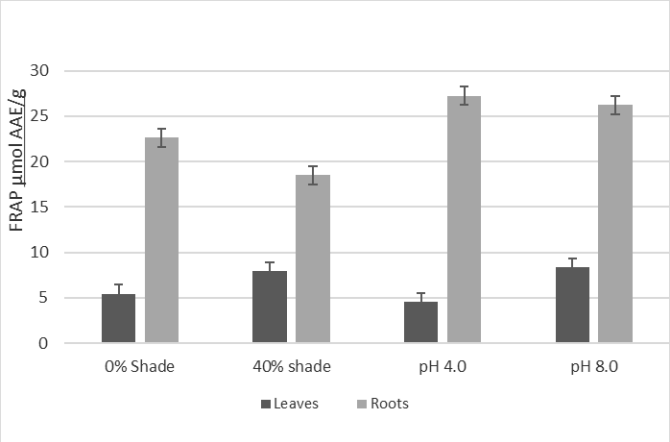

Fig. 4: The antioxidant activity Frap (µmol AAE/g) of the leaves and roots of *T. violacea* plants under different light intensities and pH levels (4 and 8) (n = 18).

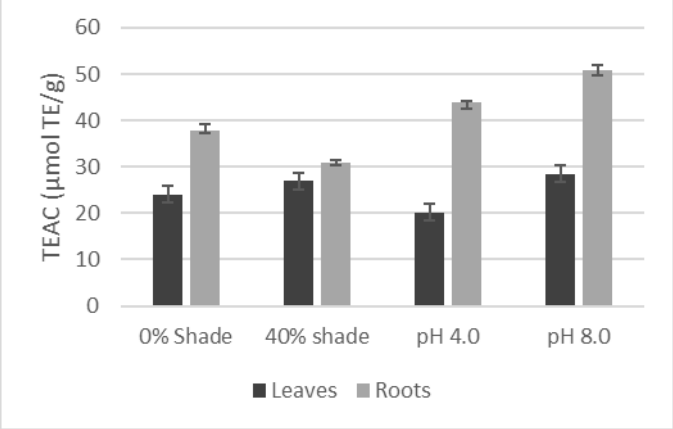

Fig. 5: The antioxidant activity (TEAC [µmol TE/g]) content of the leaves and roots of *T. violacea* plants under different light intensities and pH 4 and 8 (n = 18).

IV. DISCUSSION

A. Light intensity

Light intensity and pH are among the most important environmental factors that influence a plant's basic physiological processes, such as photosynthesis, respiration, transpiration, and carbohydrates [24].

In this study, the effects of light on both antioxidants-content and -capacity in the leaves and bulbous roots of *T. violacea* varied with treatment. The polyphenol contents were markedly superior in bulbous roots of *T. violacea* at high 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 the three varieties had consistently higher values for flavonol and polyphenol content as well as a higher antioxidant activity when exposed to high irradiance (70% IR) over lower irradiance [25]. 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. [17], 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 low and high light obtained the highest ORAC in the leaves. Generally, when plants are exposed to dry and high 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 [26]. The results obtained in this study corroborate those reported by Lin et al. [27] 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 [28], [29].

The effect of pH on the antioxidant activities of the leaf varied significantly. The higher pH treatment recorded higher total polyphenol content and antioxidant activities in leaves and roots extracts of *T. violacea*. The scientific literature on the effects of different pH levels on secondary metabolites of *T. violacea* is limited and made comparisons to this study difficult. According to Daniels et al. [17] and Xie et al. [1], environmental stress factors, such as variation in soil pH, water deficit, high temperature, and shading may result in the accumulation of reactive oxygen species in plants, which in turn may increase protective secondary metabolites. Many previous studies have proved that reducing pH favors the high production of secondary metabolites [30], [31]. However, in contrast, the current study showed that high pH had a more favourable effect on polyphenol and antioxidant activities. Hence, the influence of species on the results may not be ruled out.

V. CONCLUSION

In conclusion, light intensity and pH influence secondary metabolite contents and antioxidant activities in *T. violacea*. The *T. violacea* species is rich in polyphenols, and its leaf and bulbous root extracts have good antioxidant activities. We found higher antioxidant activity in leaf extracts when *T. violacea* was cultivated under high light intensity. In contrast,

the roots showed a higher level of total flavonol content and antioxidant activities in plants exposed to low light intensity. The pH 8 was more effective in producing increased levels of antioxidants (polyphenols) and antioxidant activity (FRAP and TEAC) in the leaves when compared to pH 4. Further studies on the interactive effects of light intensity and pH on secondary metabolites and antioxidant activities are recommended.

Author Contributions: W.N., C.W.D., and FN conceived the research; W.N. conducted the research and wrote the first draft of the manuscript; C.W. Daniels and F. Nchu supervised the first author; N.G.E.R.E. reviewed and edited the manuscript; all authors read and approved the final version of the manuscript

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