Total phenols and antioxidant potential of *Pelargonium reniforme* Curtis and *Pelargonium sidoides* DC under different watering frequencies and arbuscular mycorrhiza applications

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Abstract

***Pelargonium reniforme* Curtis and *Pelargonium sidoides* DC are medicinally important plants of the large family *Geraniaceae* and indigenous to the southern areas of Africa. Both species are small shrublets or sub-shrubs whose tuberous roots contain essential metabolites used for the treatment of several diseases. The efficacy of these two species has been well documented, especially, their antioxidant potential. In this study, the antioxidant activity and accumulation of polyphenols in the root tubers of both species cultivated under varying irrigation regimes and arbuscular mycorrhiza applications were assessed using the Folin-Ciocalteu's (polyphenols), ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays. The total phenols in the water frequency treatments were found to be significantly higher in *P. sidoides* compared to that of *P. reniforme* at P≤0.05. FRAP values of *P. reniforme* were found to be significantly higher in lower water frequency (P≤0.05) with mycorrhiza addition as compared to *P. sidoides* with no significance. The results showed that the addition of mycorrhiza improved the FRAP content in the dry root tuber material of *P. reniforme.* The water frequency, mycorrhiza and their interaction showed no significant difference in the ORAC values of both species. A strong correlation was evident in the lower water frequency treatments in both species between the total polyphenols and FRAP content.**

Keywords: *Geraniaceae*, geranium oil, mycorrhiza inoculation, *Pelargonium reniforme*, *Pelargonium sidoides*

INTRODUCTION

Pelargonium reniforme Curtis and *Pelargonium sidoides* DC are medicinally important plants of the large family *Geraniaceae* and indigenous to the southern areas of Africa. About 80% of the *Pelargonium* genus are indigenous to Southern Africa with most of them endemic to the south-western Cape province (Lalli et al., 2008). Historically, Pelargoniums have been commonly used as medicinal plants, with many of the species possessing valuable healing compounds, with studies having already proven the presence of phenolics, proanthocyanins, flavonoids, tannins and coumarins (Masondo and Makunga, 2019). The tuberous roots of *P. sidoides* are traditionally used as medicine for a wide range of ailments, in particular colds and influenza, gastrointestinal disorders, and chest pain (Schnitzler et al., 2008). Both *P. reniforme* and *P. sidoides* are widely used by traditional healers in certain parts of South Africa and Lesotho (Latté and Kolodziej, 2004). The medicinal value of these species has accelerated their harvesting in the wild, which threatens their existence. The commercial production is a viable alternative to protect and conserve these important species (Ingarfield et al., 2019). However, there are still very few commercial farmers of these species across south Africa. This is due to limited research on their cultivation requirements and water-use efficiency. Various

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provinces in south Africa are still experiencing water scarcity and as such, clean irrigation water is directed to farming of edible crops. This then call for innovative techniques to cultivate these crops using less water without compromising yield.

Arbuscular mycorrhiza (AM) symbiosis has been reported to render several mechanisms to cope with the negative effect of drought stress in plants. This fungus improves water uptake, changes soil water retention properties, increases plant gas exchange and water use efficiency in plants under drought stress (Attarzadeh et al., 2019). AM has also been reported to enhance plant growth in *Antirrhinum majus* (Asrar et al., 2012) and *P. graveolens* under water deficiency stress (Amiri et al., 2017). Likewise, the inoculation of AM in *Dracocephalum moldavica* enhanced the antioxidative enzymes, thus decreasing reactive oxygen species which damages plant cells causing a reduction in plant growth (Ghanbarzadeh et al., 2019). Therefore, there is a need to understand the effect of drought stress and AM inoculation in *P. reniforme* and *P. sidoides* to lay a commercial production protocol for these species in regions where drought is of major concern. Hence, this study was conducted to evaluate the effect of different watering frequencies and AM applications on total phenols and antioxidant potential of *P. reniforme* and *P. sidoides*.

MATERIALS AND METHODS

Experimental location and plant preparation

The experiment was conducted in the research greenhouse of the Department of Horticultural Sciences at the Cape Peninsula University of Technology, Cape Town, South Africa, located at 33°55'58.27"S; 18°25'57.04"E. The research greenhouse was equipped with environmental control with temperatures set to range from 21 to 26°C during the day and 12-18°C at night. The average humidity was maintained at 60-70% during the experimental period. *P. reniforme* and *P. sidoides* plant material were obtained from the Kirstenbosch Botanical Gardens, Cape Town, South Africa. 300 uniform tip cuttings were made as described by Ingarfield et al. (2019), with each plant having 150 cuttings. Once rooted, 100 *P. reniforme* and 100 *P. sidoides* plants of uniform size were transplanted into 12.5-cm pots containing an inert, sterilized media (v:v 2:1:1:1) made up of coco peat, foamalite, Consol® silica sand grade 6/17, and perlite. The plants were then harden-off for a week under a shade cloth before they were moved to the experimental site.

Experimental design and treatments

The experiment consisted of five watering regimes (WR) which were applied to 50 plants per species without the inoculation of AM- and to 50 plants per species with inoculation of AM+. A factorial experiment with 10 replicates having 10 individual plants per species was used to study the effects of various irrigation intervals with AM+ and without AM- on *P. reniforme* and *P. sidoides.* A total of 320 mL of water was applied to each pot based on Wang et al. (2003) research, drip irrigation was utilized with the following irrigation intervals per pot: 1) WF1 (320 mL once a day); 2) WF2 (320 mL once every 3 days); 3) WF3 (320 mL once every 6 days); 4) WF4 (320 mL once every 12 days); 5) WF5 (320 mL once every 24 days). The following treatments were used in the experiment: AM-, *P. reniforme* and *P. sidoides* received WF1 am-, WF2 am-, WF3 am-, WF4 am-, and WF5 am-; AM+, *P. reniforme* and *P. sidoides* received WF1 am+, WF2 am+, WF3 am+, WF4 am+, and WF5 am+. During transplanting, Mycorrhiza was inoculated with 30 g of AM (commercially available product Mycoroot™ acquired from the Horti Shop, 125 Belvedere Road, Claremont, Cape Town). Each pot received a half-strength Hoaglands solution with the following nutrients in ppm: N 210, P 31, K 235, Mg 48, Ca 200, Na 64 ppm, B 0.5 ppm, Fe 1-5, Mn 0.5, Zn 0.05, Cu 0.02 and Mo 0.01). Due to the lower nutrient requirements of native South African fynbos species, a half-strength nutrient solution was utilized.

Polyphenol and antioxidant assays

1. Sample preparation.

Harvested root materials of both species were immediately dried in a fan-drying laboratory oven at at 40°C for 7-14 days. The dried roots were ground into a fine powder using a Junkel and Kunkel model A 10 mill. Root material was extracted by mixing 100 mg of the dried powdered material with 25 mL of 80% (v/v) ethanol (EtOH) (Merck, South Africa) for 1 h. It was centrifuged at 4000 rpm for 5 min and the supernatants were used for all analyses.

2. Polyphenol assay.

Total polyphenols assay (Folin assay) was performed as described by Jimoh et al. (2019). Folin & Ciocalteu's phenol reagent (2N, Sigma South Africa) was diluted 10 times with distilled water and a 7.5% sodium carbonate (Sigma, South Africa) solution was prepared. In a 96-well plate, 25 µL of the crude extract was mixed with 125 µL Folin & Ciocalteu's phenol reagent and 100 µL sodium carbonate. The plate was incubated for 2 h at room temperature. The absorbance was then measured at 765 nm in a Multiskan spectrum plate reader (Thermo Electron Corporation, USA). The samples polyphenol values were calculated using a gallic acid (Sigma, South Africa) standard curve with concentration varying between 0 and 500 mg L^{-1}. The results were expressed as mg gallic acid equivalents (GAE) per g dry weight (mg GAE g^{-1} DW).

3. Oxygen radical absorbance capacity (ORAC) assay.

The ORAC assay was performed according to the method as described by Prior et al. (2005). 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH, peroxyl radical) (Sigma-Aldrich, South Africa) was prepared by mixing 150 mg with 6 mL of Phosphate buffer (75 mM, pH 7.4). A 48 nM fluorescein sodium salt (Sigma, South Africa) was also prepared in Phosphate buffer. Twelve µL of the crude extract was then mixed with 138 µL of the fluorescein and 50 µL AAPH in a black 96-well plate. Fluorescence conditions was set at 485 nm excitation and 530 nm emissions and the plate was read every minute for two hours using a Fluorskan Ascent plate reader (Thermo Electron Corporation, USA). Trolox (Sigma, South Africa) was used as the standard with concentrations between 0 and 25 µM Trolox. The results were expressed as µM Trolox equivalent (TE) per g dry weight (µM TE g^{-1} DW).

4. Ferric reducing antioxidant power (FRAP) assay.

The FRAP assay was performed using the method of Benzie and Strain (1999). FRAP reagent was prepared by mixing 30 mL Acetate buffer (0.3 M, pH 3.6) (Merck, South Africa) with 3 mL 2,4,6- tripyridyl-s-triazine (10 mM in 0.1 M Hydrochloric acid) (Sigma, South Africa), 3 mL Iron (III) chloride hexahydrate ($FeCl_3$ $6H_2O$) (Sigma, South Africa) and 6 mL of distilled water. In a 96-well plate, 10 µL of the crude sample extract was mixed with 300 µL of the FRAP reagent and incubated for 30 min at room temperature. The absorbance was then measured at 593 nm in a Multiskan spectrum plate reader (Thermo Electron Corporation, USA). The samples FRAP values were calculated using an L-Ascorbic acid (Sigma-Aldrich, South Africa) standard curve with concentrations varying between 0 and 1000 µM. The results were expressed as µM ascorbic acid equivalents (AAE) per g dry weight (µM AAE g^{-1} DW) (Sogoni et al., 2021).

Statistical analysis

The experimental data was analyzed using two-way analyses of variance (ANOVA), and Fisher's least significant difference was used to compare means at $P \leq 0.05$ level of significance between treatments. All calculations were done on the computer software program, STATISTICA version 13.5.0.17.

RESULTS AND DISCUSSION

Total phenols

The irrigation intervals positively affected the accumulation of polyphenols on both species, while the interaction of mycorrhiza and irrigation intervals were not significant. The highest content of polyphenols in *P. reniforme* (101.21±5.81) and *P. sidoides* (104.35±2.45) were found on WF5, while the least values (65.39±12.66 and 78.60±6.34) were observed in WF2 respectively (Table 1). These results are supported by numerous studies that have demonstrated the relationship between water availability and polyphenol production and in general the highest levels of phenolic compounds are found in plant material of plants exposed to water deficit conditions (Alinian et al., 2016; Amiri et al., 2017). Concurrently, Bogale et al. (2016) also recorded the highest phenolic compounds in tomato cultivars subjected to water stress. The decrease in antioxidants in inoculated plants as compared to non-inoculated could be ascribed to the ability of AM to improve the uptake of water and nutrients, improve tolerance of various environmental stress factors and aid in resistance of nematode damage and root diseases (Baum et al., 2015).

Table 1. The effect of watering frequencies with and without mycorrhiza on total polyphenols (mg GAE g^{-1} dry weight) in roots of *P. reniforme* and *P. sidoides*.

Treatment	*P. reniforme*	*P. sidoides*
Watering frequency		
WF1 AM+	83.70±20.27ab	87.14±10.96c
WF1 AM-	74.69±7.96bc	81.28±3.86cd
WF2 AM+	68.20±8.03c	76.59±10.59d
WF2 AM-	65.39±12.66d	78.60±6.34d
WF3 AM+	66.19±21.02cd	90.21±2.84bc
WF3 AM-	81.73±8.49b	104.16±7.36ab
WF4 AM+	68.96±8.6c	96.20±6.31b
WF4 AM-	82.08±7.45b	98.99±11.71b
WF5 AM+	84.70±14.28ab	114.70±20.14a
WF5 AM-	101.21±5.81a	104.35±2.45ab
Two-way ANOVA F-statistics		
Watering frequency	4.7*	13.3*
Mycorrhiza	2.8ns	0.0ns
Watering frequency × mycorrhiza	1.8ns	1.8ns

Mean values±SD are shown in columns. The mean values followed by different letters are significantly different at P≤0.05 (*).
ns=not significant as calculated by Fisher's least significant difference.

ORAC

Watering frequency, mycorrhiza and their interaction had no significant effect on the ORAC values of *P. reniforme*, however, marginal variability was observed in *P. sidoides* (Table 2). These results are supported by Gabriele et al. (2016) on *Sangiovese* grapevines (*Vitis vinifera* L.) where a significant increase in bioactive compounds was observed but no difference was found in antioxidant capacity.

FRAP

The watering intervals had no significant effect on the FRAP capacity on both species, while the least watering interval with mycorrhiza addition (WF5AM+) was found to be significantly higher in *P. reniforme* compared to *P. sidoides* with no significance. These results indicate that the addition of mycorrhiza improved the FRAP values in the dry root weight of *P. reniforme* (Table 3). Amiri et al. (2017) found similar results in their experiment on *P. graveolens* in which the application of AM+ resulted in an increased antioxidant scavenging capacity in the form of essential oils.

Table 2. The effect of watering frequencies with and without mycorrhiza on ORAC (µM TE g^{-1} dry weight) in roots of *P. reniforme* and *P. sidoides*.

Treatment	*P. reniforme*	*P. sidoides*
Watering frequency		
WF1 AM+	461.78±96.83a	519.13±115.16c
WF1 AM-	432.02±77.5a	613.27±138.35bc
WF2 AM+	481.95±134.66a	472.04±13.74d
WF2 AM-	422.15±100.76a	500.98±116.73cd
WF3 AM+	421.03±86.47a	517.43±77c
WF3 AM-	549.37±170.71a	632.59±133.47b
WF4 AM+	507.50±87.64a	499.91±72.86cd
WF4 AM-	425.80±29.97a	575.12±103.44bc
WF5 AM+	503.00±17.97a	678.88±162.18a
WF5 AM-	555.3±64.64a	639.57±88.67ab
Two-way ANOVA F-statistics		
Watering frequency	0.9ns	2.6ns
Mycorrhiza	0.0ns	2.5ns
Watering frequency × mycorrhiza	1.6ns	0.6ns

Mean values±SD are shown in columns. The mean values followed by different letters are significantly different at P≤0.05 (*). ns=not significant as calculated by Fisher's least significant difference.

Table 3. The effect of watering frequencies with and without mycorrhiza on FRAP (µM AAE g^{-1} dry weight) in roots of *P. reniforme* and *P. sidoides*.

Treatment	*P. reniforme*	*P. sidoides*
Watering frequency		
WF1 AM+	700.69±160.4bc	696.28±81.78d
WF1 AM-	575.02±49.75cd	782.98±84.96bc
WF2 AM+	739.70±212.04b	760.19±84.96bc
WF2 AM-	538.06±184.84d	699.39±134.05d
WF3 AM+	613.31±96.34c	798.06±282.69bc
WF3 AM-	581.41±77.91cd	931.70±165.55a
WF4 AM+	781.15±205.84ab	762.65±98.72bc
WF4 AM-	740.72±116.9b	721.00±72.2c
WF5 AM+	798.41±104.93a	854.33±111.8b
WF5 AM-	748.14±65.17b	916.32±142.11ab
Two-way ANOVA F-statistics		
Watering frequency	2.6ns	2.4ns
Mycorrhiza	4.2*	0.7ns
Watering frequency × mycorrhiza	0.5ns	0.7ns

Mean values±SD are shown in columns. The mean values followed by different letters are significantly different at P≤0.05 (*). ns=not significant as calculated by Fisher's least significant difference.

CONCLUSIONS

Total phenols and ORAC capacity of both species inoculated with mycorrhiza gradually increased with less irrigation intervals, although these values were not significantly different from one another. This study demonstrated that there is significant advantage of inoculating mycorrhiza to mitigate water stress in the cultivation of these two medicinal species. This is especially important in the light of water scarcity which is a major concern in South Africa. These results could be helpful to commercial farmers of the species in provinces where drought is pertinent.

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