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Short communication

Smoke–water stimulates secondary metabolites during *in vitro* seedling development in *Tulbaghia* species

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ABSTRACT

Tulbaghia species (Alliaceae) are well-known for their medicinal, horticultural and ornamental potential. The current study investigated the effect of varying smoke–water (SW) dilutions on *in vitro* germination, seedling growth and phytochemical content in *Tulbaghia ludwigiana* and *Tulbaghia violacea*. The mean germination time (MGT) was considerably reduced by approximately 2-fold in SW (1:500) treatment when compared to the control. High germination (≥80%) percentage was observed in all cases; however, there was no significant difference in germination (%) with and without SW. At post-germination stage, previous treatment with SW (1:500) produced more roots in *T. ludwigiana* seedlings. There was no significant improvement in growth parameters in SW-treated *T. violacea* seedlings. *T. ludwigiana* treated with SW (1:500) had significantly higher phenolic, flavonoid and condensed tannin contents when compared to the control. However, SW had no stimulatory effect in the level of phenolic, flavonoid and condensed tannin observed in *T. violacea* seedlings. These findings suggest the potential of SW treatment in enhancing the aforementioned phytochemical content in *in vitro T. ludwigiana* plants, which could be important during *ex vitro* growth and resultant biological efficacy of this widely used plant.

1. Introduction

Globally, the increasing popularity and use of medicinal plants is well-known. The recent surge in demand for medicinal plants has been attributed to their therapeutic potential and abundant secondary metabolite reservoirs (Lubbe and Verpoorte, 2011). Although the numerous benefits derived from plant species cannot be overemphasized, the effect of incessant harvesting, mainly manifested in the depletion of wild populations, remains a major concern (Jäger and Van Staden, 2000; Affolter and Pengelly, 2007). It is imperative to devise means to guarantee a continuous supply of renewable resources (Canter et al., 2005). As a possible solution, effective propagation and better understanding of the physiology of medicinal plants could alleviate pressure on wild population and provide sufficient plant material for local and global markets (Jäger and Van Staden, 2000).

During cultivation, challenges such as low germination rates and unpredictability of phytochemical content of plant species are major concerns (Canter et al., 2005). Therefore, researchers are channelling more efforts towards overcoming these problems. The stimulatory effect of supplements such as smoke–water (SW) on the germination of several plant species is well-recognized (Kulkarni et al., 2011). Recently, it has also been established that SW increases the phytochemical contents in plants (Aremu et al., 2012; Kulkarni et al., 2013).

The medicinal, ornamental and horticultural potential of the genus *Tulbaghia* (Alliaceae) is well documented (Aremu and Van Staden, 2013). As with other important medicinal plants, over-harvesting of wild plants will eventually affect the natural populations, leading to potentially serious declines. Thus the protection of the genus *Tulbaghia* is of paramount interest to researchers (Vosa, 2007). Although *Tulbaghia* species are easy to grow and are popular garden plants, there is inadequate information on the seed germination and potential means of enhancing secondary metabolite production which could help guarantee their conservation and successful commercialization (Sparg et al., 2005). Often, increased phytochemicals in plants with medicinal potential is directly associated with their efficacy. We evaluated the role of SW treatment on *in vitro* germination, seedling growth and phytochemical content in *T. ludwigiana* Harv. and *T. violacea* Harv.

2. Materials and methods

2.1. Source of smoke–water (SW) and Tulbaghia seeds

Stock SW solution (from *Themeda triandra* leaf material), as prepared by Baxter et al. (1994), was used in the current study. *T. violacea* and *T. ludwigiana* seeds were purchased in 2010 from Silverhill Seed Nursery, Cape Town and African Bulbs, Napier, South Africa, respectively. Prior to experimentation, the seeds were stored in plastic containers and maintained at 10 °C.

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Table 1
Effect of smoke–water on germination of *Tulbaghia ludwigiana* and *Tulbaghia violacea*.

Treatment	*Tulbaghia ludwigiana*				*Tulbaghia violacea*			
	MGT (days)		Germination (%)		MGT (days)		Germination (%)	
Control	2.9 ± 0.24	b	80.0 ± 7.70	a	2.1 ± 0.78	a	97.9 ± 2.08	a
SW 1:500	1.6 ± 0.43	a	82.2 ± 11.76	a	3.3 ± 0.55	a	97.9 ± 2.08	a
SW 1:1000	3.1 ± 0.52	b	91.1 ± 2.22	a	2.9 ± 0.26	a	93.8 ± 3.99	a
SW 1:1500	2.3 ± 0.07	ab	86.7 ± 6.67	a	3.2 ± 0.33	a	95.8 ± 2.41	a

Mean value (±SE, where n = 4) in same column with different letter(s) indicates significant differences ($p \leq 0.05$) based on Duncan's Multiple Range Test. SW = smoke–water; MGT = mean germination time.

2.2. Tests for seed viability, moisture content and imbibition

The viability, moisture content and imbibition of both *Tulbaghia* species were determined as previously described (International Seed Testing Association, 1999). For each experiment, four replicates of 25 seeds each *Tulbaghia* species were used. Seed viability was determined using 2,3,5-triphenyl tetrazolium chloride (TTC) solution. Seeds were placed on Petri dishes on filter paper moistened with distilled water. After 24 h, 1% TTC was added to the seeds in the Petri dishes and incubated in the dark at room temperature (25 ± 2 °C) for 24 h. Seeds were classified as viable if embryos were stained red. Moisture content was determined by placing seeds in an oven at 110 °C. Once a constant mass was achieved, the moisture content of the seeds was determined. For imbibition tests, the seeds were placed in Petri dishes with two layers of filter paper (Whatman No.1) moistened with 5 ml distilled water and allowed to imbibe at room temperature. At 3 h intervals, for 48 h, the seeds were blotted dry, weighed and then returned to the wet filter paper. The amount of water imbibed by the seeds was expressed as a percentage increase over the initial seed mass.

2.3. Decontamination of seeds and germination experiments

For decontamination, the seeds were soaked in 70% ethanol for 60 s followed by 0.2% fungicide (Benlate®, Du Pont de Nemours Int., South Africa) for 2 min and 3.5% commercial bleach for 5 min. The seeds were then thoroughly rinsed with distilled water. To enhance germination, seeds were imbibed in water for different times (0, 6, 12, 24 and 48 h). Based on preliminary trials, 24 h water imbibed seeds were used in the current study. Thereafter, the seeds were germinated *in vitro* on 1/10th strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with varying SW dilutions (0, 1:500, 1:1000, 1:1500). Media were autoclaved at 121 °C for 20 min and dispensed at 30 ml per Petri-dish. Four replicates of 15 seeds were

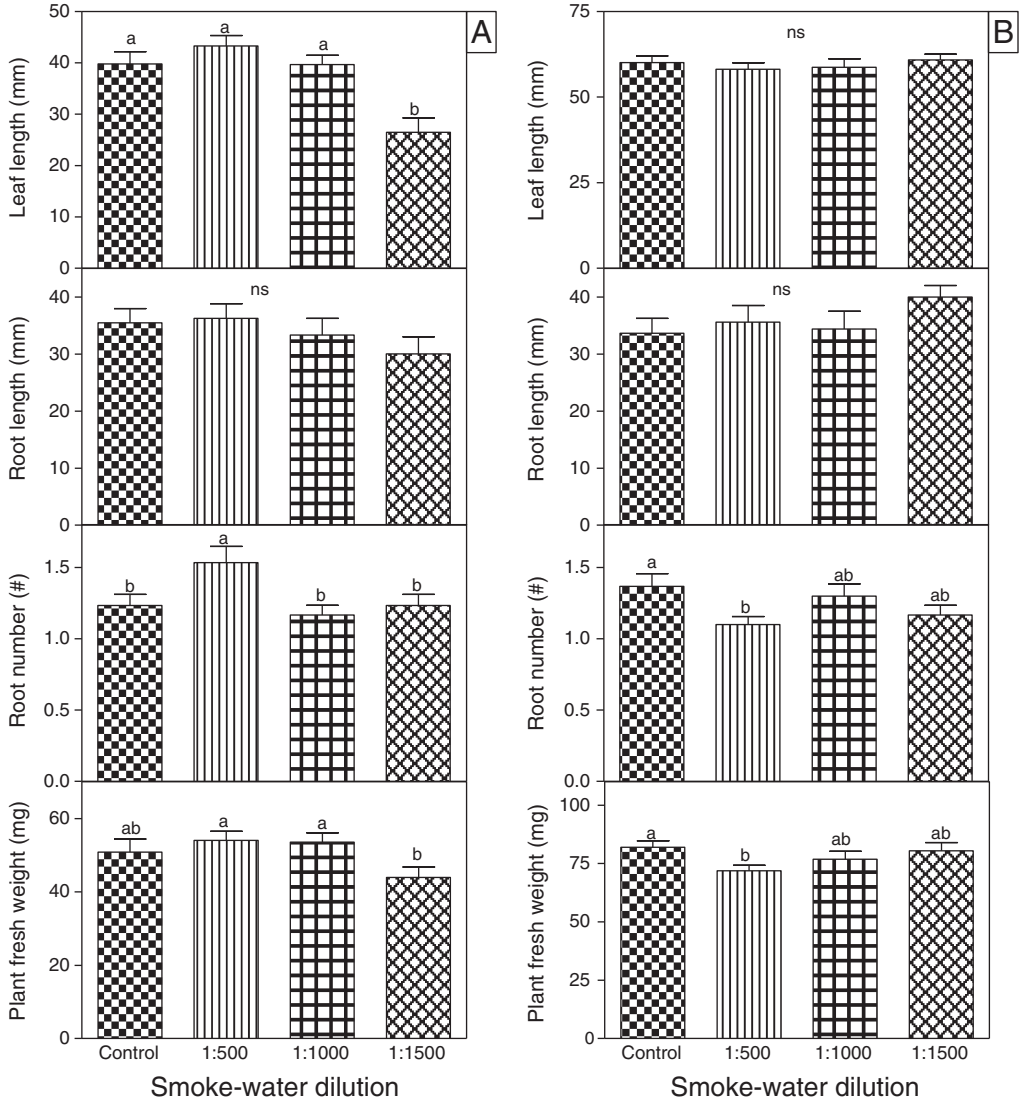

Fig. 1. Effect of smoke–water (SW) on the growth of *Tulbaghia ludwigiana* (A) and *Tulbaghia violacea* (B) 15-day-old *in vitro* seedlings. For each graph, bar (mean value ± SE and n = 30) with different letter(s) indicates significant differences ($p \leq 0.05$) based on Duncan's Multiple Range Test. ns = not significant.

used for each SW treatment or control. *Tulbaghia* species were cultured under 24 h light conditions with a photosynthetic photon flux (PPF) of 45 μmol m^{-2} s^{-1} at 25 ± 2 °C. A seed was considered as germinated when the radicle protruded 2 mm. Germination counts for all experiments were made daily for 15 days. Mean germination time (MGT) was calculated as outlined by Ellis and Roberts (1981). After 15 days, seedlings were harvested and the growth parameters were recorded.

2.4. Phytochemical content quantification

Phytochemical contents in the 15-day-old seedlings of *Tulbaghia* species were determined using colorimetric methods. Oven-dried whole plant material (100 mg) was ground and extracted with 10 ml of 50% methanol in an ice-bath for 20 min and filtered using Whatman No. 1 filter paper. Thereafter, phenolics, flavonoids and condensed tannins were quantified (Aremu et al., 2012). Total phenolics, flavonoids, condensed tannins and iridoids were expressed in mg gallic acid equivalents (GAE), mg catechin equivalents (CE), and μg or mg cyanidin chloride equivalents (CCE) per g DW, respectively. Three replicates were used for the phytochemical assays.

2.5. Data analysis

Data were subjected to one-way analysis of variance (ANOVA) using the SPSS software package for Windows (SPSS®, version 10.0 Chicago, USA). Where there was statistical significance ($p \leq 0.05$), the mean values were further separated using Duncan's Multiple Range Test.

3. Results and discussion

3.1. Seed viability, moisture content and imbibition

Both *Tulbaghia* species demonstrated seed viability of ≥95%. The moisture content of *T. violacea* (approximately 41%) was higher than that for *T. ludwigiana* seeds (31%). *T. ludwigiana* seeds generally had better absorption compared to *T. violacea*. After 72 h a fresh mass of approximately 200% and 140% was observed for *T. ludwigiana* and *T. violacea*, respectively.

3.2. Effect of smoke–water treatments on germination of Tulbaghia species

While SW at 1:500 dilution enhanced the MGT in *T. ludwigiana*, there was no noticeable positive effect for *T. violacea* (Table 1). Both species demonstrated high (≥80%) germination which is in agreement with previous findings reported for *T. violacea* (Kulkarni et al., 2005; Sparg et al., 2005). Generally, SW had no significant stimulatory effect on the observed germination (%) for both *Tulbaghia* species. Prior imbibition of seeds before application of SW treatment has been documented to reduce germination (Light et al., 2002). This may explain the slight decline in germination observed in *T. violacea*.

3.3. Effect of smoke–water on growth of in vitro Tulbaghia seedlings

In addition to the effect of SW during germination, its role during the post-germination phase has been documented in several species (Kulkarni et al., 2011). Often, the effect of SW is more apparent in roots compared to shoots (Aremu et al., 2012). A significant improvement was observed in root number of *T. ludwigiana* seedlings treated with SW (1:500; Fig. 1A). Generally, SW treatments had no significant

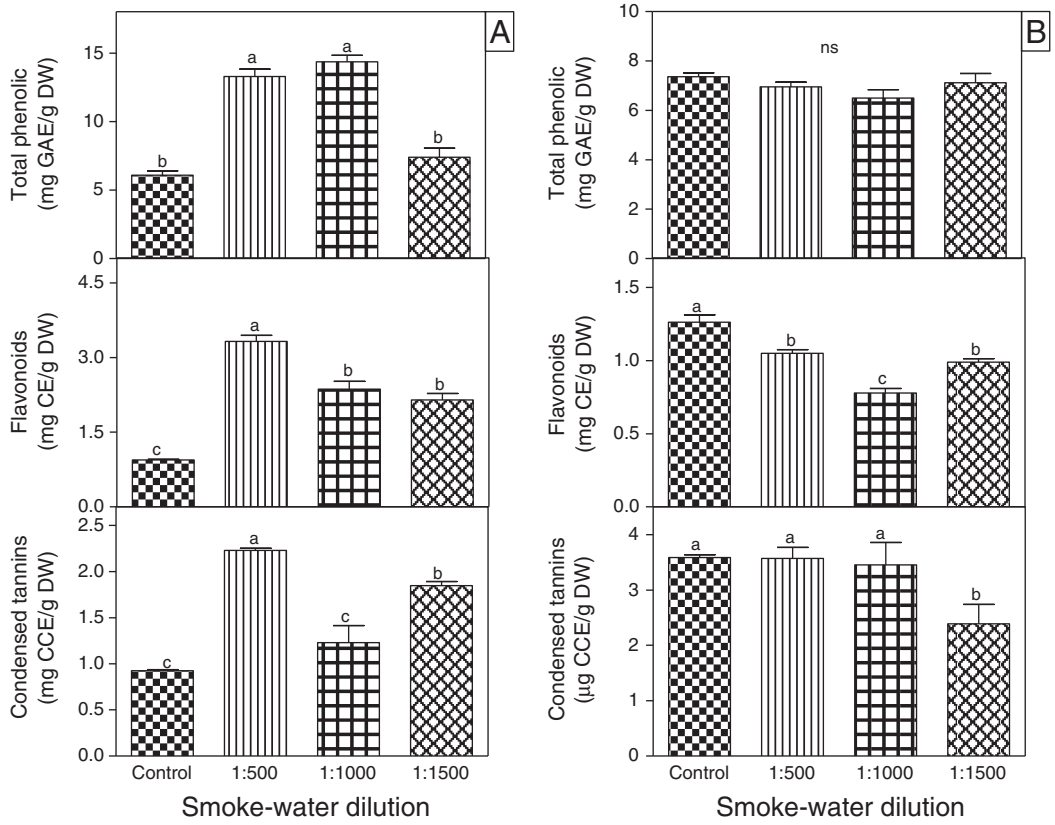

Fig. 2. Effect of smoke–water (SW) on the phytochemical content in *Tulbaghia ludwigiana* (A) and *Tulbaghia violacea* (B) 15-day-old *in vitro* seedlings. GAE = Gallic acid equivalents, CE = Catechin equivalents, CCE = cyanidin chloride equivalents, DW = dry weight. For each graph, bar (mean value ± SE and n = 6) with different letter(s) indicates significant differences ($p \leq 0.05$) based on Duncan's Multiple Range Test.

stimulatory effect on the growth parameters in *T. violacea* seedlings. Nevertheless, it was evident that SW (1:1000 and 1:1500) had no detrimental effect on the growth of *T. violacea* seedlings as the recorded values compared favourably with the control. Likewise, Kulkarni et al. (2013) observed that different concentrations of SW had no significant stimulatory effect on *Aloe arborescens* seedlings.

3.4. Effect of smoke–water treatment on phytochemical content in Tulbaghia species

Smoke–water significantly enhanced the phenolic, flavonoid and condensed tannin contents in *T. ludwigiana*, while it had no stimulatory effect in *T. violacea* (Fig. 2). Phenolic content was highest (14 mg GAE/g DW) in *T. ludwigiana* seedlings treated with SW (1:1000). Flavonoids and condensed tannins were highest in SW-treated (1:500) *T. ludwigiana*. The level of phytochemicals in plants is often associated with resultant biological activity and their survival (Alain-Michel, 2007). Thus, the ability of SW to significantly increase the levels of phenolics, flavonoids and condensed tannins in *T. ludwigiana* is valuable. Recently, the stimulatory effect of SW on some phytochemicals (*e.g.* indigo, phenolics and flavonoids) in several species has been reported (Zhou et al., 2011; Aremu et al., 2012; Kulkarni et al., 2013). Based on molecular evidence, it has been established that SW can up-regulate the phenylpropanoid-pathway and flavonoid-related genes thereby enhancing phenolic biosynthesis (Soós et al., 2010). In the current study, phenolic content in SW-treated (1:1000) *T. ludwigiana* was approximately 2-fold higher than in the control seedlings. Similarly, about a 2.4-fold higher level of flavonoids was detected in *T. ludwigiana* supplemented with SW (1:500) compared to the control. The function of secondary metabolites such as phenolic compounds in plant survival is well known as they act as signalling molecules (Peer and Murphy, 2007), as well as for defence against plant herbivores and pathogens (Bennett and Wallsgrove, 1994).

4. Conclusions

Generally, SW treatment had no significant effect on the germination percentage and MGT of both *Tulbaghia* species; however, there was a favourable effect during the post-germination stage whereby root number of *T. ludwigiana* was increased. Phenolics, flavonoids and condensed tannins in *T. ludwigiana* were also higher following treatment with SW (1:500). Generally, SW had no stimulatory effect on the growth and investigated phytochemicals in *T. violacea*. Thus, it is apparent that the response of plant species to SW is species-dependent. The current findings suggest a potential avenue for enhancing the cultivation/conservation *via* increased phytochemicals in the investigated species. However, it will be valuable to evaluate the role of SW on the status of other well-known constituents (*e.g.* cysteine S-oxides) in *Tulbaghia* species. Based on the fact that 15-day-old seedlings were tested, further studies focusing on greenhouse-based and field trials (with older plants) will be necessary to substantiate the current findings.

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