In Vitro Germination and Initial Seedling Development of Krantz Aloe by Smoke-saturated Water and Seed Imbibition

Claudia A. Espinosa-Leal¹ and Silverio Garcia-Lara¹

ADDITIONAL INDEX WORDS. *Aloe arborescens*, germination pretreatment, KAR₁, karrikinolide, seed germination, seed water soaking

SUMMARY. Krantz aloe (*Aloe arborescens*) is one of two aloe species currently used for the extraction of active ingredients that can be useful in the cosmetic and pharmaceutical industries. However, krantz aloe plants have been frequently collected from the wild, which is threatening wild populations. In vitro tissue culture would allow the growth of krantz aloe under controlled conditions, optimizing the production of active ingredients without risk to wild populations. The establishment of cultures from krantz aloe plant explants has proved difficult as a result of the long response times of the explants and their release of polyphenols. Krantz aloe seeds are not commonly used as a means of propagation because of their low germination percentages. The objective of this study was to evaluate the effects of seed imbibition (SI) with water and the addition of smoke-saturated water (SSW) to the culture medium on the in vitro germination and initial seedling development of krantz aloe. Seeds were germinated in vitro in axenic conditions. The treatments used were the addition of 10% SSW to the culture media, SI, and a combination of both (10% SSW + SI). Daily germination was recorded and gross morphology was measured after 1 month of culture establishment. The maximum germination percentage (GP) was achieved when 10% SSW was in the medium (97.2%), followed by the combined application of 10% SSW + SI (96.6%), both of which were almost 30% greater and significantly different from that of the control (69.4%). SI had an 83.3% GP. No significant differences were observed among treatments in any of the observed development parameters (leaf and root length and number). Taken together, these findings show that the use of SSW improves the in vitro germination of krantz aloe without affecting seedling development, indicating preliminarily that SSW represents a useful in vitro germination promoter for this species.

True aloe (*Aloe vera*) and krantz aloe (*Aloe arborescens*) are currently being used for the extraction of cosmetic and nutraceutical active ingredients (Cardarelli et al., 2017; Espinosa-Leal and Garcia-Lara, 2019). Krantz aloe has a wide geographical distribution in the African continent, with populations in South Africa, Botswana, Swaziland, Lesotho, Mozambique, Zimbabwe, Mapaura and Timberlake, and

Malawi. Although krantz aloe is common within its range, human activity has caused a negative impact on populations (Smith et al., 2008). Wild populations of aloe species are currently threatened as a result of their continuous collection for transplantation to private gardens or the extraction of active ingredients (Maundu et al., 2006). Conventional propagation using lateral shoots and rhizome cuttings is not able to fulfill the increasing market demand for aloe (Cristiano et al., 2016).

In vitro tissue culture represents a promising alternative to wild collections of krantz aloe plants and

conventional propagation by allowing the production of multiple plants for their reintroduction to the wild and fields, and production of active metabolites to be optimized (Espinosa-Leal et al., 2018). Although some reports exist on the in vitro tissue culture of krantz aloe (Bedini et al., 2009; Cardarelli et al., 2017; Kawai et al., 1993), the establishment of cultures from plant explants has proved difficult because of the long response times of the explants and their release of polyphenols, resulting in the need for constant subcultures (Bedini et al., 2009).

Seeds are an alternative explant for in vitro culture establishment. True aloe seeds are not commonly used as a means of propagation (traditional and in vitro) because of their scarcity in nature and low GPs of 0% to 25% (traditional) and 60% to 70% (in vitro) (Cristiano et al., 2016). Studies have investigated the optimization of germination conditions for bitter aloe (*Aloe ferox*) and krantz aloe in petri dishes without substrate and with filter paper, which have shown that the use of karrikinolide (KAR₁)-rich SSW either as a priming treatment or a watering solution promotes seed germination (Bairu et al., 2009; Kulkarni et al., 2013). Studies have demonstrated that SSW can also promote seedling vigor, including leaf and root length and number (Demir et al., 2018). Hydration of seeds before sowing is another method for improving germination (Demir et al., 2018; Khan, 1992). However, the effect of SSW and SI, separately and combined, on the in vitro germination of krantz aloe under aseptic conditions using Murashige and Skoog (MS) culture media (Murashige and Skoog, 1962) as a standard substrate has not been investigated previously. Therefore, the objective of this work was to evaluate the effect of SI with water and the addition of SSW to the culture media on the in

Received for publication 1 May 2020. Accepted for publication 3 June 2020.

Published online 27 July 2020.

¹BioScience Unit, EIC, Tecnológico de Monterrey, Campus Monterrey, Avenue Eugenio Garza Sada 2501, Monterrey, N.L., 64849, México

This work was supported by grants from the Research Nutriomics Chair Funds and CAT-005 of Tecnológico de Monterrey, as well as postdoctoral fellowships presented to C.A.E.-L. by CONACyT, Mexico, and Tecnológico de Monterrey.

C.A.E.-L. is the corresponding author. E-mail: claudia_espinosa@tec.mx.

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<https://doi.org/10.21273/HORTTECH04643-20>

Units

To convert U.S. to SI, multiply by	U.S. unit	SI unit	To convert SI to U.S., multiply by
29.5735	fl oz	mL	0.0338
0.3048	ft	m	3.2808
2.54	inch(es)	cm	0.3937
25.4	inch(es)	mm	0.0394
28.3495	oz	g	0.0353
(°F - 32) ÷ 1.8	°F	°C	(°C × 1.8) + 32

vitro germination and initial seedling development of krantz aloe.

Materials and methods

Seeds (152 total) were obtained from the mature fruit (eight total) of two different 4-year-old krantz aloe plants growing at an altitude of 2300 m (lat. 19.483°N, long. 98.903°W) provided by Bioimpulsora Company (Texcoco, Mexico) in Mar. 2018. The seeds were stored at 4 °C until the start of the experiment on 12 Apr. 2018.

An SSW solution was prepared according to the method described by Coons et al. (2014), with some modifications—namely, the reduction of the initial plant cellulose. Briefly, 36.5 g of filter paper (No. 1; Whatman, Little Chalfont, UK) was cut into strips and placed inside a bee smoker (Mieles Tecnología, Merida, México), filling it to three-quarter capacity. The paper at the bottom of the smoker was then ignited and covered with more paper until smoke was produced. A heat-resistant hose was attached to the opening of the bee smoker and was connected to a Kitasato flask containing 500 mL distilled water, and a pressure pump-inducing vacuum was used to draw the smoke through the water. The bellows on the bee smoker were pumped occasionally to allow for the continuation of combustion. As the filter paper burned, more was added to the smoker until the initial sample was fully combusted. The system was then allowed to cool for 15 min. The saturated smoke solution was transferred to an amber bottle and stored in a refrigerator at 4 °C until later use (about 1 month).

The germination experiments were performed three times independently with seeds from the same batch of fruit. Because of the scarcity of seeds, each experiment consisted of four treatments that used four glass jars (capacity, 110 mL) each and a final treatment with 12 seeds per treatment. The treatments were as follows: treatment 1, 10% SSW and 1:10 MS 10% SSW; treatment 2 (SI), seeds imbibed in distilled water for 16 h (SI) and placed in 1:10 MS; and treatment 3 (10% SSW + SI), seeds imbibed in water for 16 h and placed in 1:10 MS 10% SSW. As a control, seeds were placed in 1:10 MS.

The seeds were surface-disinfected using the following procedure. Seeds

were washed under running water, submerged in a soap–water 1% (by volume) liquid detergent (Salvo PA00186821; Procter & Gamble, Cincinnati, OH) and 0.5% (by volume) nonionic surfactant (Tween® 20; Hycel, Zapopan, México) for 15 min, washed in sterile distilled water, immersed in a 15% (v/v) commercial sodium hypochlorite (Clorox, Oakland, CA) solution for 20 min, and finally washed with sterile distilled water under a laminar flow hood (Purifier class II bioseft cabinet; LABCONCO, Kansas City, MO). Three seeds were then placed in each glass jar with 20 mL 1:10 (w/w) MS (M5519; Merck, Darmstadt, Germany) (Bairu et al., 2009; Murashige and Skoog, 1962) with 7% agar (A296; PhytoTechnology Laboratories, Shawnee Mission, KS) using either distilled water or 10% SSW as a solvent.

Cultures were placed in a controlled-environment chamber (Signature Diurnal Growth Chamber model 2015; VWR International, Radnor, PA) at 25 °C with a 12:12-h photoperiod [12 h of light at 89.1 μmol·m⁻²·s⁻¹ (Dual-Range Traceable Light Meter; Control Co., Friendswood, TX) and 12 h of complete darkness]. The seeds were examined every 24 h for 15 d. Germination was considered complete when the seedcoat had broken and a radicle of ≈2 mm in length had emerged (Bairu et al., 2009). Germination initiation (GI) was counted when at least one of the seeds germinated; germination end (GE) was considered as the last day

a seed germinated for each treatment. At the end of the experiment, the GP (Scott et al., 1984), coefficient of velocity of germination [CVG (Nichols and Heydecker, 1968)], and germination rate index [GRI (Esechie, 1994)] were calculated as shown in Eqs. [1], [2], and [3] respectively:

$$GP = (n_k)100/N, \quad [1]$$

where n_k is the final number of seeds germinated in a lot and N is the total number of seeds;

$$CVG = \left(\sum_{i=1}^k f_i / \sum_{i=1}^k f_i x_i \right) 100, \quad [2]$$

where f_i is the number of seeds newly germinated on day i , x_i is the number of days from sowing, and k is the last day of germination;

$$GRI = \sum_{i=1}^k (GP_i)100/x_i, \quad [3]$$

where GP_i is the germination percentage on day i , x_i is the number of days from sowing, and k is the last day of germination

The numbers of leaves and roots were counted, and the length of the longest leaves and roots was measured using a Vernier caliper. Measurements were recorded 1 month after the initial culture establishment (Bairu et al., 2009). Data were analyzed using one-way analysis of variance, followed by Tukey's test when a significant

Table 1. Germination parameters of in vitro culture of krantz aloe in 1:10 Murashige and Skoog culture media with a 12-h (light) photoperiod and at a 25 °C (77.0 °F) constant air temperature in a controlled-environment chamber.

Treatment ^z	CVG ^y	GRI (%/d) ^x	GI (d) ^w	GE (d) ^v
	(mean ± SD)			
C	13.5 ± 2.3 NS	46.6 ± 3.9 NS	6.3 ± 0.9 NS	12.7 ± 7.3 NS
10% SSW	13.1 ± 1.6 NS	63.2 ± 18.6 NS	5.7 ± 0.5 NS	9.7 ± 1.7 NS
SI	16.7 ± 3.0 NS	73.2 ± 8.6 NS	5.0 ± 0.0 NS	7.3 ± 1.7 NS
10% SSW + SI	15.4 ± 3.0 NS	77.9 ± 15.4 NS	5.3 ± 0.5 NS	8.3 ± 2.1 NS

^zC = control; SSW = 10% smoke-saturated water; SI = seed imbibition.

^yCoefficient of velocity of germination (CVG) = $\left(\sum_{i=1}^k f_i / \sum_{i=1}^k f_i x_i \right) 100$, where f_i is the number of seeds newly germinated

on day i , x_i is the number of days from sowing, and k is the last day of germination.

^xGermination rate index (GRI) = $\sum_{i=1}^k (GP_i)100/x_i$, where GP_i is the germination percentage on day i , x_i is the number of days from sowing, and k is the last day of germination.

^wFirst day of germination (counted when at least one of the seeds germinated).

^vLast day of germination (the last day a seed germinated for each treatment).

GI = germination initiation; GE = germination end.

NS, Nonsignificant at $P < 0.05$ via ANOVA ($n = 36$).

difference was found. All data processing and analyses were conducted using Minitab Express (Minitab, State College, PA) with a 5% significance level.

Results and discussion

No significant difference was found between the treatments with regard to the timing of GI or GE (Table 1). Bairu et al. (2009) found previously that the in vitro germination of bitter aloe was very erratic, with germination starting on day 10 and ending more than 1 month after sowing. By contrast, krantz aloe presented shorter times to GI and GE. A study by Demir et al. (2018) found that seed priming with SSW produced a uniform germination in pepper (*Capsicum annuum*) seeds, both mature and immature. SSW and/or KAR₁ was involved in the breaking of seed dormancy and the promotion of embryonic development (Demir et al., 2018; van Staden et al., 2006).

The only germination parameter affected by the treatments was GP (Fig. 1). The GP, 10% SSW and 10% SSW + SI were significantly greater than the control, whereas SI was similar to the control, 10% SSW, and 10% SSW + SI. The maximum GP obtained (mean $\pm$ SD) for 10% SSW was 97.2% $\pm$ 4.8%, followed by 10% SSW + SI (96.7% $\pm$ 5.8%), both of which were almost 30% greater than the control (69.4% $\pm$ 4.8%) (Fig. 1). Several studies have demonstrated that smoke can promote the in vivo germination of many different species of plants, including bitter and krantz aloe (Bairu et al., 2009; Kulkarni et al., 2013). The application of SSW as a watering solution in petri dishes resulted in 80% GP in bitter aloe (Bairu et al., 2009) and 60% GP in krantz aloe (Kulkarni et al., 2013), both of which were greater than control levels. In our study, a GP of more than 95% was achieved in the treatments that included SSW. We suspect these high levels of GP, compared with the previous studies, may be explained by the greater concentration of SSW used (10% in comparison with 0.2% used before).

The SI did not improve the in vitro germination of krantz aloe, which is in agreement with the findings of Santini et al. (2017) for two cactus species (Cactaceae). Because

imbibition improves and synchronizes germination by hydrating the seeds to begin the preliminary process of germination (Demir et al., 2012; Khan, 1992), this finding suggests that the humidity conditions were sufficient to remove the need for prior seed hydration, as experienced by Gupta et al. (2020), who found that seeds exposed to room temperature formed a thin layer of water that initiated the process of germination. Seedcoat structure is a determinant of the need for seed imbibition before soaking. In general, a permeable or “soft” seedcoat absorbs water more

easily, opposite to more impermeable or “hard” seedcoats that do not imbibe water even after days of soaking (Ma et al., 2004; Shao et al., 2007; Varela and Alborno, 2013). Krantz aloe seedcoat can then be classified as permeable. No other germination parameters were affected by the treatments in our study.

A representative photograph of krantz aloe seedlings after 30 d is shown in Fig. 2. No significant difference was found in the gross morphology of the seedlings among treatments (Table 2). Initial seedling development was unaffected by the

Fig. 1. Effects of seed imbibition (SI) and smoke-saturated water (SSW) on the in vitro germination percentage of krantz aloe in 1:10 Murashige and Skoog culture media with a 12-h (light) photoperiod and at a 25 °C (77.0 °F) constant air temperature in a controlled-environment chamber; C = control, 10% SSW = addition of 10% smoke saturated water to culture media, SI = seed imbibition for 16 h. Data are expressed as mean $\pm$ SD. Values with different letters are significantly different via Tukey’s test at $P < 0.05$ ($n = 36$).

Table 2. Gross morphology of in vitro krantz aloe plantlets after 1 month of initial culture establishment in 1:10 Murashige and Skoog culture media with a 12-h (light) photoperiod and at a 25 °C (77.0 °F) constant air temperature in a controlled-environment chamber.

Treatment ^z	Leaves (no.)	Roots (no.)	Leaf length (cm) ^y	Root length (cm)
	(mean $\pm$ SD)			
C	2.7 $\pm$ 0.5 NS	3.4 $\pm$ 0.7 NS	1.4 $\pm$ 0.4 NS	1.2 $\pm$ 0.4 NS
10% SSW	2.5 $\pm$ 0.7 NS	3.4 $\pm$ 1.4 NS	1.1 $\pm$ 0.3 NS	0.7 $\pm$ 0.6 NS
SI	2.6 $\pm$ 0.5 NS	2.7 $\pm$ 1.4 NS	1.3 $\pm$ 0.5 NS	0.9 $\pm$ 0.6 NS
10% SSW + SI	3 $\pm$ 0.4 NS	3.1 $\pm$ 0.9 NS	1.3 $\pm$ 0.3 NS	0.7 $\pm$ 0.5 NS

^zC = control; 10% SSW = 10% smoke-saturated water; SI = seed imbibition.

^y1 cm = 0.3937 inch.

NS, Nonsignificant at $P < 0.05$ via analysis of variance ($n = 36$).

Fig. 2. Representative photograph of krantz aloe seedling after 1 month of growth in vitro in 1:10 Murashige and Skoog culture media with a 12-h (light) photoperiod and at a 25 °C (77.0 °F) constant air temperature in a controlled-environment chamber; C = control, 10% SSW = addition of 10% smoke saturated water to culture media, SI = seed imbibition for 16 h.

addition of SSW to the culture medium, which contrasts with the findings of Chumpookam et al. (2012), who discovered that seedlings of papaya (*Carica papaya*) treated with 1% to 10% SSW exhibited significant increases in all growth parameters compared with the control. Bairu et al. (2009) found previously that bitter aloe seedlings develop better in the presence of culture medium containing low salt concentrations (1:10 MS culture media). Therefore, it is possible the addition of SSW to the culture medium altered the solute concentration, preventing any improvement in overall seedling growth for krantz aloe. However, we did not measure the salt content of the culture media.

Krantz aloe is a commercially important plant that is becoming increasingly threatened in the wild. The species is currently included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2019) Appendix II, and is considered vulnerable in certain regions and occurs in protected areas within its range (Martínez Richart, 2019). Hence, a need exists for the development of new cultivation methods to ensure the continued availability of its products. The addition of SSW to the culture medium improved the in vitro

GP for krantz aloe while having no effect on seedling development, suggesting it is an excellent promoter of in vitro germination for this species. Thus, SSW treatment would make the use of seeds for the establishment of in vitro tissue culture a viable option for this species; however, further work is needed to verify this report. The plantlets obtained by this process could be further exploited to obtain large-scale production of uniform, healthy plantlets that can later be used by the market (Cristiano et al., 2016; Gantait et al., 2014).

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