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Seed origin, storage conditions, and gibberellic acid on *in vitro* germination of *Campomanesia adamantium* (Cambess.) O. Berg

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***Campomanesia adamantium* (Cambess.) O. Berg seeds (guavira) are recalcitrant and rapidly lose viability upon removal from the fruit, making difficult the long-term storage. *In vitro* germination could be used as an important tool to overcome the issues related to this short viability. It might help seed conservation and species propagation. The purpose of this study was to evaluate *in vitro* germination of guavira seeds collected from different sites and stored under different conditions. Also, the sowing of these seeds in MS medium supplemented with different concentrations of gibberellic acid (GA_3) was evaluated. Seeds from the local garden were treated with: 0, 1.0, 2.0, 3.0, or 4.0 mg L^{-1} GA_3. Seeds from a local farmer's market were treated with: 0, 2.5, 5.0, 7.5 or 10 mg L^{-1} GA_3. We evaluated the germination rate, the average length of the shoot and main root, and the number of leaves. The *C. adamantium* seeds were sown immediately after harvest and treated with GA_3, regardless concentration, increased germination rate by at least 10%, whereas 1.0 mg L^{-1} GA_3 resulted in 100% germination. The shoot length increased linearly with increasing concentration of the growth regulator. Different concentrations of GA_3 had no effect on the development of the main root and leaves. Seeds acquired from a local farmer's market showed lower germination rate than those sown immediately after harvesting, and did not differ in the rate of germination under different treatments with GA_3. Furthermore, around 25% of those seedlings had abnormal leaf morphology. *C. adamantium* seeds stored at 4°C and -20°C for 60 days did not germinate successfully, suggesting that seeds under cold storage conditions cannot be used for germplasm purposes.**

Key words: Guavira, Cerrado, Myrtaceae, temperature.

INTRODUCTION

Campomanesia adamantium (Cambess.) O. Berg (guavira) is a native species to Cerrado belonging to the myrtle family Myrtaceae. The fruit is rich in phenolics compounds, with great antioxidant activity (Giada and

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Mancini Filho, 2006) and due to its sweetness and aromatic flavor it offers good market acceptance (Vieira et al., 2010). In addition to the unique flavor and nutraceutical properties of its fruit, this species presents desirable agronomic traits such as a high fruit per plant ratio (Melchior et al., 2006) and high genetic variability, which are useful for cultivar development and genetic selection (Oliveira et al., 2011).

Given these traits, *C. adamantium* is a good prospect for commercial production (Vieira et al., 2010). However, *C. adamantium* is not presently cultivated in commercial orchards, so fruits are directly harvested from natural populations. Several factors have limited the expansion of commercial orchards, such as the limited natural range of this species, susceptibility to insects and diseases, and poor post-harvest preservation during transport and storage (Vieira et al., 2010). However, the greatest limitation is the plant (seedling) propagation due to the recalcitrance characteristics and low seed germination efficacy (Melchior et al., 2006; Scalon et al., 2012; Dresch et al., 2012). Therefore, basic researches on seed storage, germination, establishment conditions (Dresch et al., 2012), as well as the development of new and efficient asexual propagation techniques (Vieira et al., 2010) becomes critically important in order to produce seedlings, establishing a germplasm collection for preservation, and developing commercial production.

In vitro tissue culture has been successfully used for species preservation, seedling production, and selection of disease-resistant plants. *In vitro* propagation or micropropagation presents countless benefits, including rapid mass production (Moraes et al., 2007; Arrigoni-Blank et al., 2011), year-round production of uniform plants, increased biotic and abiotic stress resistance, elimination of phytosanitary problems (Moraes et al., 2007; Dias et al., 2011), and facilitation of germplasm maintenance and exchange (Braun et al., 2010). Field-grown or wild plants may not provide a suitable source for *in vitro* culture due to endogenous contamination (Soares et al., 2009). Whereas, healthy explants can be developed from vegetative material grown from seeds germinated *in vitro*. However, Oliveira et al. (2013) highlights that an embryo is the result of genetic recombination with a different genotype limiting the cloning process of superior individuals established in the field. *In vitro* germination and high quality seedling establishment (great vigor and phytossanitary conditions) may be used to initiate *in vitro* micropropagation. These techniques will assist in *C. adamantium* conservation and seedling production, avoiding the limitations imposed by propagation via seed.

In recalcitrant seeds, the lifetime is very limited, and to guarantee the species reproduction favorable environmental conditions for germination are required. In this sense, Dresch et al. (2012) found that the maintenance of high moisture content of *C. adamantium* seeds is essential. The same authors found that at 25°C, seeds submitted to drying for 16 h (27% water content) followed by storage for 18 days lose their germinating power (0%) in relation to the newly processed seeds with 57% moisture content (52%), evidencing the sensitivity to desiccation.

Seed germination is a complex process, controlled by both physical and internal regulating factors, and in this sense, GA plays very important role in controlling and promoting germination, stem elongation and meristmetic tissue development (Gupta and Chakrabarty, 2013). The application of gibberellic acid, although do not influence the recalcitrance of seeds, plays an important role in stem or internode elongation, stimulating cell division and expansion, promoting favorable conditions for the germination and seedlings establishment.

For all these reasons, our research objective was to evaluate the efficiency of *in vitro* germination of *C. adamantium* seeds collected from different sites, and, although the seeds are classified as recalcitrant, seed germination stored without desiccation was evaluated, maintaining the high moisture content, under different conditions and treated with different gibberellic acid concentrations.

MATERIALS AND METHODS

Fruits *C. adamantium* (Camb.) O. Berg were harvested in November 2013 from plants grown in the Garden of Medicinal Plants at the Faculdade de Ciências Agrárias, Universidade Federal da Grande Dourados, Mato Grosso do Sul (MS), Brazil. In November 2014, fruits were obtained from a farmer's market in the Dourados city (MS).

The fruits from plants grown in the garden were processed immediately after harvest, while the fruits from a farmer's market were processed after three days of harvest, which were kept by the farmer at favourable environmental conditions. The fruits were pulped and the seeds were separated under running water. In a laminar flow cabinet, seeds were rinsed with 70% ethanol, immersed in 2.5% sodium hypochlorite for 5 min, washed three times in sterilized water, and superficially dried with sterilized filter paper. After this procedure and in order to sterilize external surfaces seeds were used in one of five experiments designed under a completely randomized design. In the first two experiments, the initial germination capacity of the seeds was immediately evaluated. For the other experiments, the seeds were stored in brown paper bags for 60 days at one of the following temperatures: ±25°C (environmental conditions maintained by air conditioning), 4°C, or −20°C. After storage, seed surfaces were sterilized a second time as previously described. All experiments tested the effects of different concentrations of gibberellic acid (GA_3) on germination efficacy.

In the first experiment, seeds from the local garden were treated with different GA_3 concentrations: 0 (control), 1.0, 2.0, 3.0, or 4.0 mg L^{-1}. Each of the five treatments included four replicates of three culture flasks with seven seeds each. In the other experiments seeds from a local farmer's market were treated with different GA_3 concentrations: 0 (control), 2.5, 5.0, 7.5, or 10 mg L^{-1}. Each of the five GA_3 treatments included five replicates of one culture flask with five seeds each. In every experiment seeds were sownin 260 mL glass culture flasks containing 30 mL of MS culture medium (Murashige and Skoog, 1962), with 30 g L^{-1} of sucrose, 100 mg L^{-1} of myo-inositol, 6 g L^{-1} of agar, and the specified concentration of

Table 1. Analysis of variance (ANOVA) summary for the *in vitro* germination of *Campomanesia adamantium* seeds extracted from fruit collected in the local garden.

SV	DF	MS			
		G	ALS	ALMR	ALL
GA_3 concentration	4	0.13*	0.65^{ns}	0.14^{ns}	0.01^{ns}
Residue	12	0.03	0.29	0.10	0.02
VC (%)		11.6	12.3	17.3	7.9
OA		96.3	4.4	1.8	2.4

**, * and ns, significant at 1 and 5% and non-significance, respectively, by the test F. SV, Source of variation; DF, degrees of Freedom; SM, mean square; VC, variation coefficient; OA, overall average. Germination - G (%), Germination of normal plants (GNP) and abnormal (GAP); ALS, Average length of shoots (cm); ALMR, Average length of main root (cm); ALL, average number of leaves.

Table 2. Analysis of variance (ANOVA) summary for the *in vitro* germination of *C. adamantium* seeds extracted from fruit exposed at the local farmer's market.

SV	DF	SM					
		G	GNP	GAP	ALS	ALMR	ALL
GA_3 concentration	4	0.04^{ns}	0.16^{ns}	0.14^{ns}	0.17^{ns}	0.03^{ns}	0.48^{ns}
Residue	16	0.05	0.10	0.12	0.07	0.13	0.17
VC (%)		30.5	69.5	79.5	20.9	28.2	29.3
OA		49.6	26.1	24.3	1.2	1.2	1.8

**, * and ns, significant at 1 and 5%, and non-significance, respectively, by the test F. SV, Source of variation; DF, degrees of Freedom; SM, mean square; VC, variation coefficient; OA, overall average. Germination - G (%), Germination of normal plants (GNP) and abnormal (GAP); ALS, Average length of shoots (cm); ALMR, Average length of main root (cm); ALL, average number of leaves.

GA_3, with the final pH adjusted to 5.8. The medium was sterilized in an autoclave for 20 minutes at 121°C, under 1.5 atm of pressure.

After sowing, the flasks were transferred to a growth chamber set at 25 ± 2°C. Seeds from the garden were subjected to an initial 15-day period of darkness, while seeds from the market were kept in darkness for 7 days; after this period, all seeds were grown under light with a photosynthetic photon flux density of 45 µmol m^{-2} s^{-1} and a photoperiod of 14 h.

After day 45, seeds from the local garden fruit were scored for percent of germination, average length of the shoot (cm), average length of the main root (cm), and average number of leaves. After day 30, seeds from the farmer's market fruit were scored for germination rates (total = seeds that developed roots; normal = seedlings with root and leaf; abnormal = seedlings with stem axis but no leaves), average length of shoot (cm), average length of the main root (cm), and average number of leaves.

Percentage data were transformed to arcsine values, while count and continuous data were transformed to square root prior ANOVA analysis. Means were compared by polynomial regression using the statistical software package Winstat (Machado et al., 1999).

RESULTS AND DISCUSSION

According to the ANOVA different concentrations of gibberellic acid (GA_3) showed a significant effect (p=0.05) on *in vitro* germination of seeds collected from the garden and immediately inoculated after harvest (Table 1). Farmer's market fruit seeds did not show a significant response to different concentrations of GA_3 (Table 2).

Seeds stored at ambient temperature (approximately 25°C) germinated prematurely and became contaminated with fungi during storage, probably due to the combination of relatively high humidity and temperature, precluding their use in further experiments. The seeds stored at 4°C and −20°C for the period of 60 days lost their germinative power, with no germination observed 30 days after *in vitro* sowing. Our results corroborated those of Melchior et al. (2006) and Scalon et al. (2009), where the authors showed that guavira seeds presented recalcitrance, lost germinative power during storage, and only germinated successfully when obtained from fruits right after their harvest and pulping.

Garden seeds sown right after harvest presented significant differences in percent germination when treated with different concentrations of GA_3. The addition of GA_3 to the culture medium, regardless of concentration, increased the percent of germination by approximately 10% over the control. Maximum germination was observed at 2.48 mg L^{-1} GA_3 (calculated value) (100%) (Figure 1A). For the seeds from market fruits, percent of

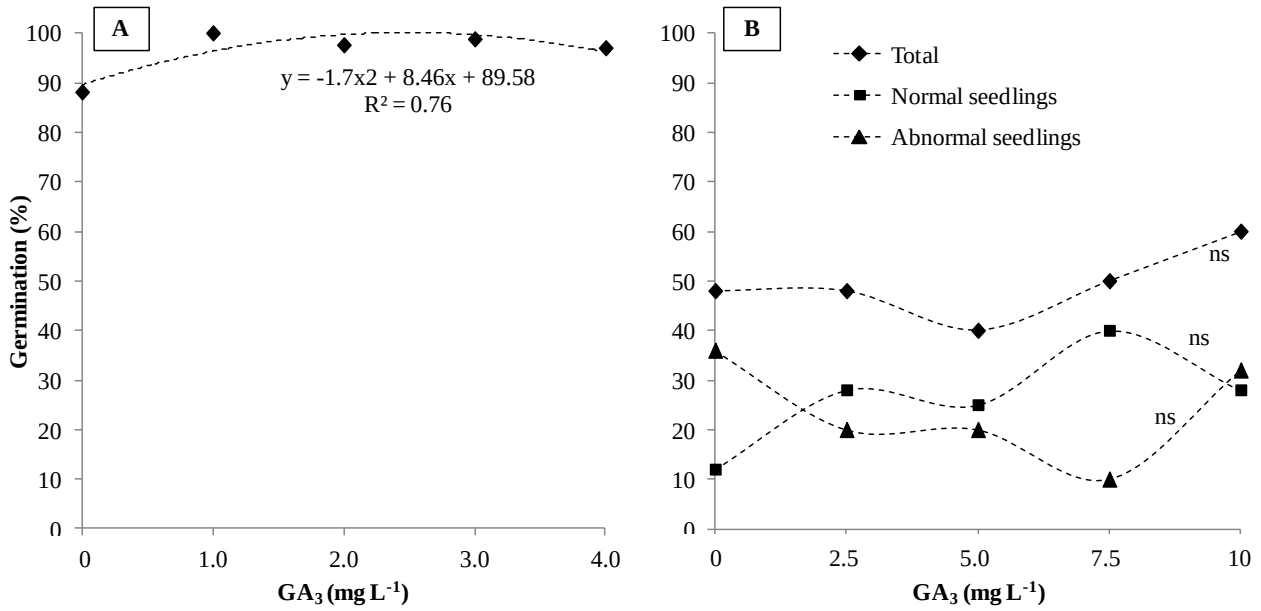

Figure 1. The effect of gibberellic acid (GA_3) in the MS medium on *in vitro* germination of *C. adamantium*. (**A)** Seeds extracted from fruit collected at the local garden. (**B)** Seed extracted from fruit exposed at the local farmer's market.

germination did not differ at different concentrations of GA_3 (Figure 1B).

In the first and second experiments, the seeds were sowing immediately after surface sterilization, but the garden and market seeds had different germination rates. These differences might be related to the amount of time between harvest and seed extraction. *C. adamantium* seeds are known to lose their viability when kept in the fruit, and they germinate most successfully right after harvest (Melchior et al., 2006). We could not establish a harvest date for the market fruits, but the seeds had already lost viability compared to those from the freshly harvested garden fruits. More research will be needed to identify the factors that lead to the reduction of viability.

Market seeds that were treated with 10 mg L^{-1} of GA_3 showed abnormal morphology in approximately 25% of the seedlings (Figure 1B), with the primary leaves atrophied or undeveloped. The development incomplete or abnormal of seedlings may be attributed to the fruit harvest time and storage conditions. The harvest date and storage conditions prior to selling might affect factors that are essential for proper seedling development.

In the polynomial regression using garden seeds, shoot length demonstrated a linear response to the concentration of GA_3 (Figure 2A). The long shoots (4.8 cm) grew in the culture medium with 4.0 mg L^{-1} of GA_3. In market seeds, different concentrations of GA_3 did not affect shoot length over the control; average seedling length was 1.2 cm (Figure 2B). Main root development was not influenced by gibberellic acid at any concentration in any of the experiments (Figure 2A and B). Soares et al. (2012) reported a positive influence of GA_3 on the shoot length in the orchid *Dendrobium nobile* Lindl. They also observed that plant height increased linearly with the increase of GA_3 concentration, suggesting a correlation between the two. According to Santos et al. (2013), gibberellin use may inhibit or minimize the impact of adverse factors in the quality and performance of seeds, and gibberellins increase the speed at which seeds emerge and aid in seedling development. During *in vitro* propagation, species that are sensitized by gibberellic acid elongate more rapidly and can be transferred from culture more quickly, allowing efficient production of large numbers of robust individual plants (Alcantara et al., 2014).

Simões et al. (2012) studied the effect of gibberellins, at the same concentrations we tested with the garden seeds, during *in vitro* germination of long pepper (*Piper hispidinervum* C. DC.), and contrary to our results, the authors found that shoot length responded negatively, with the longest shoots observed at the lowest concentration, 1.0 mg L^{-1} of GA_3. It is important to emphasize that growth regulators can affect cultivated species in different ways, and the classes, concentrations (Bastos et al., 2007), and the presence of endogenous phytoregulators can induce different responses in plants (Dias et al., 2008). Although *Eucalyptus dunnii* Maiden belongs to the same family (Myrtaceae) as *C. adamantium*, Navroski et al. (2013) did not find that GA_3 had a positive effect on the *in vitro* shoot elongation of this species. Treatment with GA_3 reduced germination and shoot length while increasing callus formation.

Species respond differently to gibberellins depending on tissue type, developmental stage, hormone

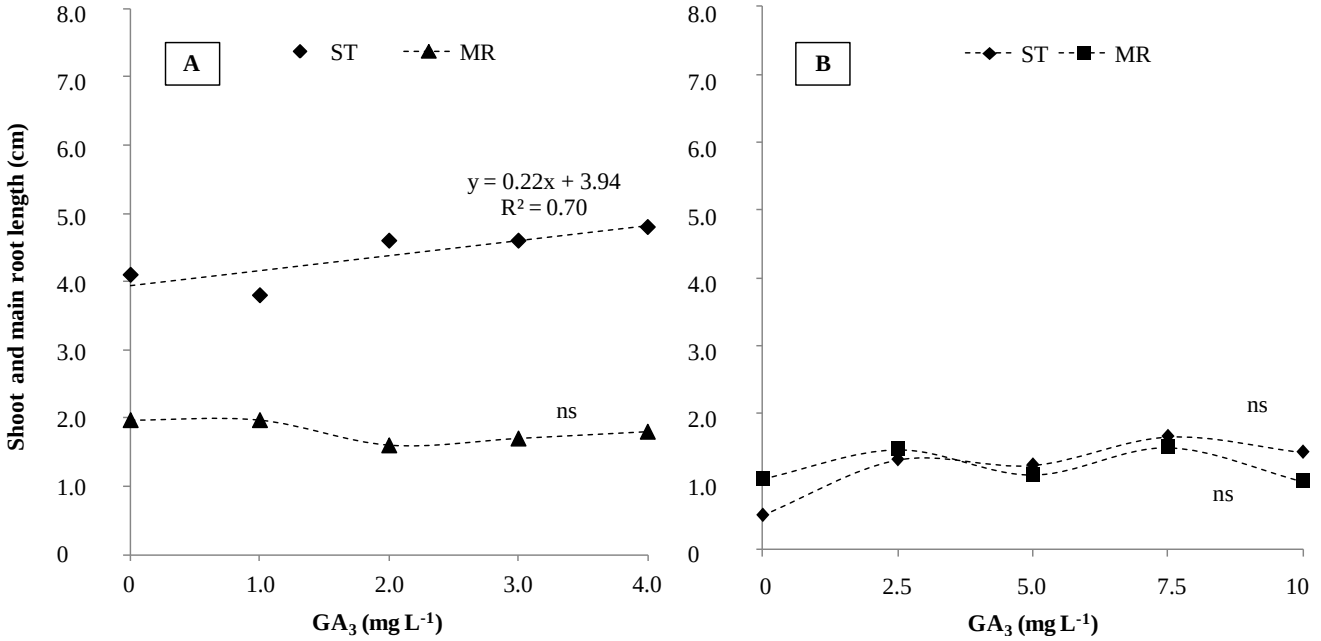

Figure 2. Average length of shoots (ST) and main root (MR) of *C. adamantium* seedlings germinated and *in vitro* cultivated in MS medium with different gibberellic acid (GA_3) concentrations. (**A)** Seeds extracted from fruit collected at the local garden. (**B)** Seed extracted from fruit exposed at the local farmer's market.

concentration, and interactions with endogenous factors. Different concentrations of GA_3 did not influence root growth, regardless of seed origin or storage treatment. Torres and Borges (2013) found similar results with *Capsicum frutescens* L. (chili pepper), with no significant difference in seedling root growth between the control and treatments with gibberellin. In contrast, Simões et al. (2012) found that the same concentrations of GA_3 that we used with the garden seeds reduced root length in long pepper (*Piper hispidinervum*) seedlings. However, according to Lima et al. (2009), in many species, including passion fruit, tangerine, soursop, and lemon trees, gibberellins promote cellular stretching and stimulate the primary root to break the tissues that restrict its growth.

We did not find a significant difference in the average number of leaves between seed types or among treatments (Figure 3A and B). However, seedlings from market seeds had a greater average number of leaves when germinated in the presence of GA_3 (Figure 3B). Machado et al. (2005) studied the effects of different concentrations of gibberellins during the acclimatization process in micropropagated rootstock of the apple cultivar Marubakaido and observed that the number of leaves was positively affected. They found that gibberellic acid induced the plants to produce a larger number of leaves by overcoming apical bud dormancy.

Garden seeds germinated right after harvest (Figure 4A) showed an increase in shoot length and leaf number in the presence of GA_3 compared to the control, although shoot length and number of leaves were not influenced by different concentrations of the regulator. Seedlings from market seeds (Figure 4B) that were germinated in a medium containing GA_3 showed better leaf blade developed, longer internodes, and a thicker main root when compared to the control.

Conclusion

The use of gibberellic acid, regardless concentration, promoted an increase of 10% in germination of seeds inoculated in a culture medium right after fruit harvest, however the use of 2.48 mg L^{-1} (calculated value) lead to 100% germination. Shoot length increased linearly as the growth regulator concentration increased. At the studied concentrations, GA_3 did not affect main root and leaf development. Seeds extracted from fruits at the farmer's market had lower a germination rate and did not respond to the different treatments with gibberellic acid. The seeds had an elevated percentage (±25%) of seedlings with abnormal leaf morphology. Seeds stored at 4°C and −20°C for 60 days did not germinate, whereas seeds stored at ambient temperature (±25°C) germinated prematurely. These storage conditions are not suitable for seed conservation.

Conflict of Interests

The authors have not declared any conflict of interests.

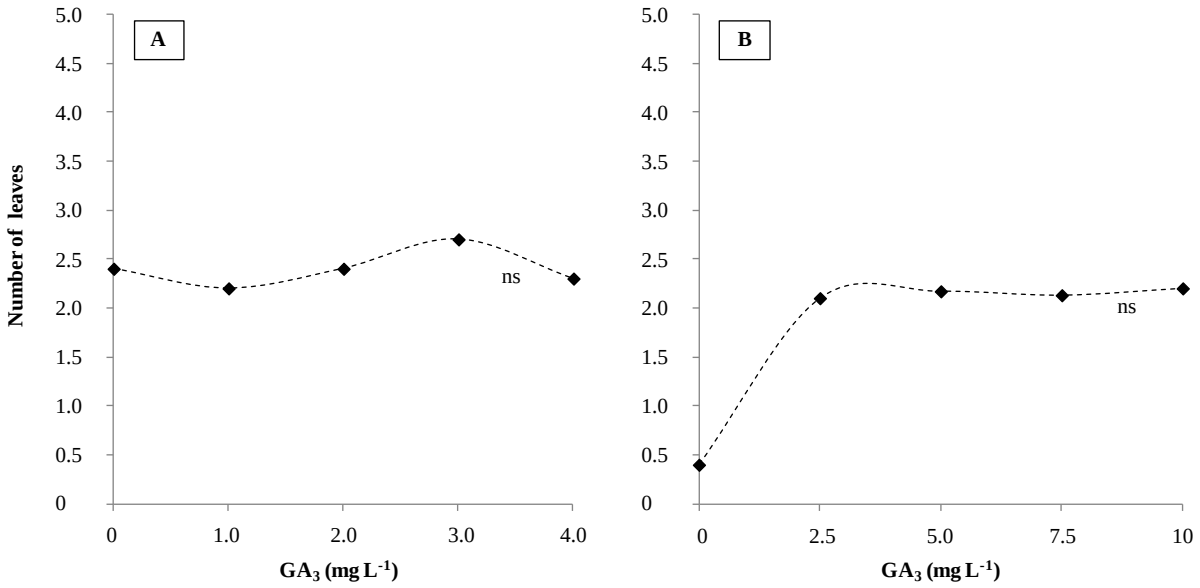

Figure 3. Average number of leaves of *C. adamantium*, seedlings germinated and *in vitro* cultivated in MS medium with different gibberellic acid (GA_3) concentrations. (**A)** Seeds extracted from fruit collected at the local garden. (**B)** Seed extracted from fruit exposed at the local farmer's market.

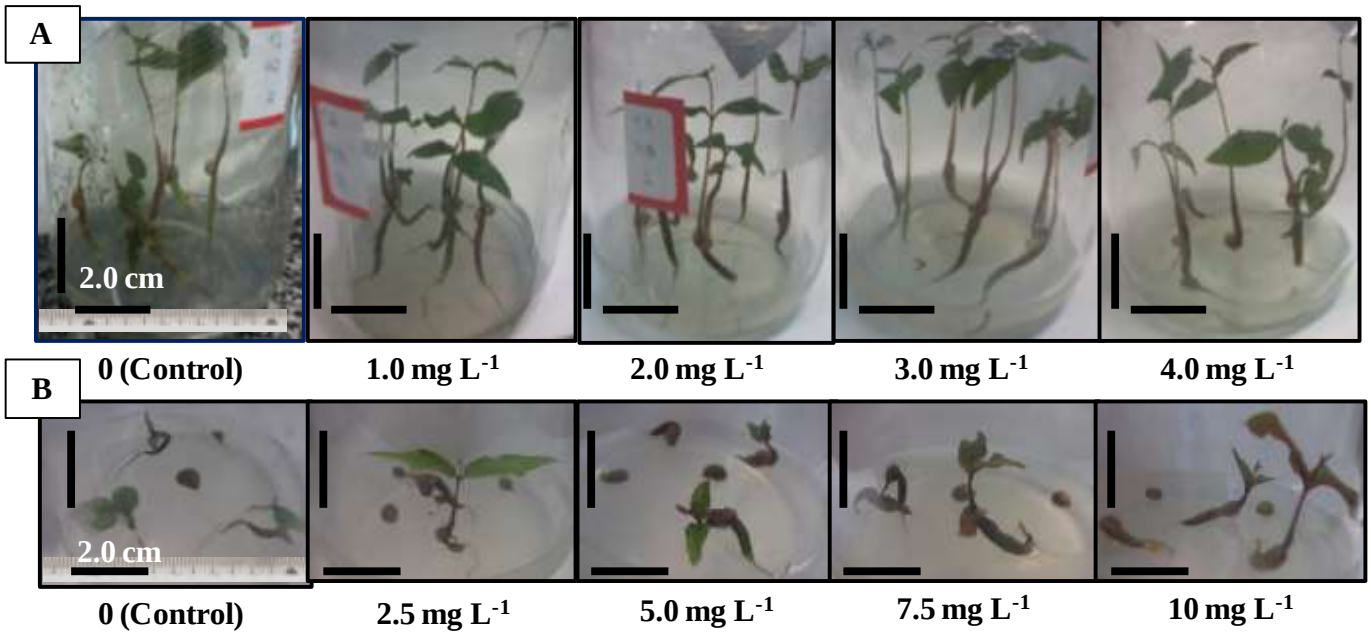

Figure 4. General appearance of *C. adamantium* seedlings germinated and *in vitro* cultivated in MS medium with different gibberellic acid (GA_3) concentrations. (**A)** Seeds extracted from fruit collected at the local garden. (**B)** Seed extracted from fruit exposed at the local farmer's market.

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