In vitro propagation of *Zingiber spectabile* [1]

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ABSTRACT

Zingiber spectabile is a tropical ornamental species with difficulties to obtain efficient propagation system. Thus, this study aimed to assess the *in vitro* propagation of *Zingiber spectabile*. Seed characterization was determined by measuring length, width and thickness, the weight of 1000 seeds and imbibition curve. *In vitro* germination of seeds was at constant (25 °C) or alternating temperatures (20-30 ºC). For optimization of *in vitro* multiplication, different concentrations of activated charcoal (0.0, 0.1 and 0.3%) and sucrose (0.0, 0.1, 0.3, 0.5 and 0.7 M) were evaluated. Plantlets were inoculated in flasks with different sealing systems (PVC covers with or without filters at the center) and culture media (MS or WPM). The plants were acclimatized in Plantmax® substrate. Seeds were of 6.06 mm length, 3.22 mm wide and 2.83 mm thick. The weight of 1,000 seeds corresponded to 46.4 g. The seed imbibition curve approaches to a tree phase pattern. Alternating temperatures induced high germination rates (68%). The addition of 0.3% activated charcoal provided higher root growth and plants with smaller number of senescent leaves. The best plant growth was obtained by the use of 0.1 M sucrose. All acclimatized plants survived (100%). The results demonstrate that *Z. spectabile* respond well to *in vitro* propagation.

Keywords: beehive ginger, micropropagation, tropical plants.

RESUMO
Propagação *in vitro* de *Zingiber spectabile*

Zingiber spectabile é uma espécie ornamental tropical com dificuldades para se obter um sistema de propagação eficiente. Assim, este estudo teve como objetivo avaliar a propagação *in vitro* de *Z. spectabile*. A caracterização das sementes foi determinada pela medição do comprimento, largura e espessura, pelo peso de 1.000 sementes e pela curva de embebição. A germinação *in vitro* das sementes foi avaliada em temperatura constante (25 °C) ou alternada (20-30 °C). Para a otimização da multiplicação *in vitro,* diferentes concentrações de carvão ativado (0,0, 0,1 e 0,3%) e sacarose (0,0, 0,1, 0,3, 0,5 e 0,7 M) foram avaliadas. As plântulas foram inoculadas em frascos com diferentes sistemas de vedação (tampas de PVC com ou sem filtros no centro) e meio de cultura (MS ou WPM). As plantas foram aclimatizadas em substrato Plantmax®. As sementes apresentaram comprimento de 6,06 mm, largura de 3,22 cm e espessura de 2,83 mm. O peso de 1000 sementes correspondeu a 46,4 g. A curva de embebição de sementes se aproxima de um padrão trifásico. Temperaturas alternadas induziram altas taxas de germinação (68%). A adição de 0,3% de carvão ativado proporcionou maior crescimento radicular e plantas com menor número de folhas senescentes. O melhor crescimento de plantas foi obtido pelo uso de sacarose 0,1 M. Todas as plantas aclimatizadas sobreviveram (100%). Os resultados demonstraram que *Z. spectabile* responde bem à propagação *in vitro*.

Palavras-chave: sorvetão, micropropagação, plantas tropicais.

1. INTRODUCTION

As an attractive tropical ornamental plant, *Zingiber spectabile* (Zingiberaceae) is widely cultivated in Brazil due its high commercial value. This species is known as beehive ginger or ornamental ginger, and is used for landscaping and as a cut flower (VIÉGAS et al., 2012; LESSA et al., 2015).

Besides ornamental uses *Z. spectabilie* has others important applications: leaves and rhizomes are used for culinary purposes and for the preparation of traditional medicines (SADHU et al., 2007). Rhizomes are rich in phenolic compounds with antioxidant and antibacterial properties, which can be used as a natural preservative in the food industry (SIVASOTHY et al., 2012a; 2013). Zerumbone is the most abundant component in the rhizome, which has been found to possess multiple biomedical properties, such as antiproliferative, antioxidant, anti-inflammatory, and anticancer activities (SIVASOTHY et al. 2012a; RAHMAN et al. 2014). A dimeric flavonol glycoside (Spectaflavoside A), a potent iron chelating agent, is also found in *Z. spectabile* rhizomes (SIVASOTHY et al., 2012b).

Despite its wide use and economic importance, large-scale propagation methods are not well established for this species. *Z. spectabile* is commonly propagated by plant division or by rhizomes, which are not efficient methods for commercial propagation of quality planting materials (because of possible transmission of diseases and very small number of plants obtained). This species is susceptible to several pests and diseases, nematodes and bacteria, which may be easily transmitted by conventional methods of propagation (BEZERRA and LOGES, 2005). Therefore, the use of tissue culture techniques as micropropagated plants of *Z. spectabile* is highly recommended to produce a healthy planting stock in large scale.

Tissue culture techniques have been reported as an efficient method to several species of *Zingiber,* as *Z. officinale, Z. rubens, Z. moran* and *Z. zerumbet* (HAMIRAH et al., 2010; MOHANTY et al., 2011; ABBAS et al., 2011; DAS et al., 2013; SERAN, 2013; YEPTHOMI and MAITI, 2014; NIRMAL BABU et al., 2016). However, there are not completed established protocol to *in vitro Z. spectabile,* just few studies in some steps of micropropagation of this species are available (FARIA and ILLG, 1995; OLIVEIRA et al., 2010; MOREIRA et al., 2012; NERY et al., 2015). In none of these studies were used seed to *in vitro* established and propagation.

Therefore, this study aimed to determine seed characteristics and protocol for *in vitro* propagation (seed germination, growth conditions and acclimatization) of *Z. spectabile* to produce large quantities of quality plants for the ornamental market.

2.   MATERIALS AND METHODS

Seeds characterization and germination

Seeds of *Z. spectabile* with black color (mature seed) were randomly selected from a population of 30 plants. The moisture content of seeds was determined by drying the seed in oven at 105 ± 2 ºC for 24 hours (BRASIL, 2009). Three replicates were used with 10 seeds each.

A digital caliper (accurate to 0.5 mm) was used to measure length (longitudinal distance between the apex and the base - cm), width (measured perpendicularly to the middle region of the seeds - cm) and thickness of the seeds.

The weight of 1000 seeds was determined using eight replicates of 100 seeds, weighed on analytical balance accurate to 0.001 g, according to Brasil (2009). Seed coats were subjected to manual cross-section cuts using a scalpel. Sections were stained by a mixture (9:1) of Astra Blue (1% in water) and Safranin (1% in water), mounted in 1:1 (v/v) glycerol: water, and sealed with nail polish (KRAUS and ARDUIN, 1997). The seeds were cut transversely to determine the type of reserve composition, staining with solution of lugol to indicate the presence of starch granules in cells (KRAUS and ARDUIN, 1997). The provisional and semi-permanent blades were assembled following the techniques described by Johansen (1940). Observations were made with the use of an Axiophot microscope equipped with DIC optics (Zeiss®, Oberkochen, Germany) with photographic

documentation carried out using a Powershot A 640 digital camera (Canon®, Tokyo, Japan).

The seed imbibition curve was determined with use of four replicates of 25 seeds each (BRASIL, 2009), inside in crystal polystyrene boxes (Gerbox®) with Germtest® paper substrate moistened with distilled water in the amount of 2.5 times the dry paper mass (BRASIL, 2009). The seeds were stored in a B.O.D. (Biochemical Oxygen Demand) incubator under constant light 36 μmol m^{-2} s^{-1} irradiancy, provided by fluorescent lamps (20 W, Osram, Brasil) at a temperature of 30 °C. Initially, the seeds were weighed every 30 minutes during the first 8 hours of imbibition by means of an analytical precision scale (0.0001 g). After this time, the seeds were weighed at intervals of three hours, marked from the beginning of the experiment, ending the weighing after the primary root protrusion of 50% of seeds. At every weighing, the seeds were removed from the Gerbox® and placed over paper to absorb external moisture, weighed and then returned to the Gerbox® and B.O.D. The percentage increase over the initial fresh weight was determined.

To evaluate the germination rate, seeds were placed on MS medium (MURASHIGE and SKOOG, 1962) with 0.7% agar (w/v), 0.09M sucrose (w/v), pH 5.8, and maintained at 100% relative humidity. Germination tests were conducted in B.O.D. either at constant temperature (25 °C) or alternating temperatures (20-30 ºC) during night and day, respectively, constant light with photosynthetic photon flux density of 36 μmol m^{-2} s^{-1}, provided by fluorescent lamps (20 W). The germination parameter was evaluated after 30 days.

Germination was assessed using the root protrusion at ± 2.0 mm as criteria (MAGUIRE, 1962). The germination speed index (GSI) was also determined and calculated according to the equation proposed by Maguire (1962). The treatments were arranged in a completely randomized design (CRD) with three replications of 10 seeds per treatment.

In vitro multiplication

Nodal segments of *Z. spectabile in vitro* established was used as explants for axillary shoot induction in MS medium supplemented with 4 mg L^{-1} 6-benzyladenine (BA), 0.09 M sucrose (w/v) and 0.7% agar (w/v), pH 5.8 (NERY et al., 2015). The material was stored in B.O.D. under 16-hours photoperiod light, photosynthetic photon flux density of 36 μmol m^{-2} s^{-1} provided by fluorescent lamps (20W) at a temperature of 25 ± 2 °C. After 60 days, the shoots induced were individualized and inoculated in MS medium previously described, without BA, for 15 days, for cleaning the explante of BA residues and use this explants to below experiments.

Effects of activated charcoal

Shoots of 5-6 cm from *in vitro* multiplication were inoculated in MS medium as previously described, without BA and was supplemented with different concentrations of activated charcoal (AC) 0.0; 0.1 and 0.3%. After inoculation, the shoots were transferred to a growth

chamber exposed to temperatures of 25 ± 2 °C with a 16 h photoperiod and photosynthetic photon flux density of 36 µmol m^{-2}s^{-1} provided by fluorescent lamps (20W).

The number and length of shoots, number of green or senescent leaves, number and length of root (cm), and fresh weight were measured 60 days after inoculation. The experiment was conducted in a completely randomized design and each treatment consisted of 15 shoots.

Effect of sucrose concentration

Shoots with 5-6 cm long from *in vitro* multiplication were inoculated in MS medium. The effect of different sucrose concentrations (0.0; 0.1; 0.3; 0.5 and 0.7 M) in growth medium was evaluated. After inoculation, the shoots were stored in B.O.D. under photosynthetic photon flux density of 36 µmol m^{-2} s^{-1}, temperature of 25 ± 2 °C and a 16h photoperiod.

After 60 days, shoot length (cm), root length (cm), root number, shoot number, leaf number and fresh weight (g) were quantified to determine the multiplication rate. The experiment was conducted in a completely randomized design and each treatment consisted of 15 shoots.

Effect of sealing system and medium type

Plantlets with 5-6cm from *in vitro* multiplication were inoculated in two culture media, MS and WPM (Wood Plant Medium) (LLOYD and MCCOWN, 1980), supplemented with agar 0.09 M sucrose (w/v) and 0.7% agar (w/v). In combination with type of medium, two sealing systems of flasks were evaluated; PVC (polyvinyl chloride) covers with or without filters at the center (Biosama®). All combinations were tested. The plantlets were storaged in B.O.D. under photosynthetic photon flux density of 36 µmol m^{-2} s^{-1}, temperature of 25 ± 2 °C and a 16h photoperiod.

After 60 days, shoot length (cm), root length (cm), root number, shoot number, leaf number and fresh weight (g) were quantified. The experiment was conducted in a completely randomized design, and each treatment consisted of 15 shoots.

Aclimatization

Plantlets (60 days old) with roots from *in vitro* multiplication were transferred after undergoing a period of seven days of pre-acclimatization (opening of the culture vessel), for tubes with a volume of 250 mL, containing Plantmax® and wrapped with transparent plastic bags to maintain the relative humidity in the environment. The tray with the tubes was kept in a growth chamber at a controlled temperature of 25 ± 2 °C and irradiance of 67 µm photon m^{-2} s^{-1}. At 21 days, the tubes with the plants were transferred to a greenhouse, where they remained for 10 days under 30% shading, before transplanting to black polyethylene bags containing a mixture of soil and sand at a ratio 1:1. The evaluation of plantlet survival rate was performed after 31 days. The results were analyzed using a completely randomized design with 15 replications, and each one was made up of a vase containing a plantlet.

Statistical analysis

The germination percentage was transformed in arcsine square root before the statistical analysis. All multiple comparisons were done by means of Scott-Knott test ($p \leq 0.05$) using the statistical software Sisvar® (FERREIRA, 2014).

3.   RESULTS AND DISCUSSION

Seeds characterization and germination

During the stage of seed production, we observed both senescent and non-senescent bracts at the same inflorescence. However, the seed formation occurs only on the medium bracts of senescent inflorescence (Figure 1A). Over the senescence process of inflorescence, the bracts open and the seeds are exposed.
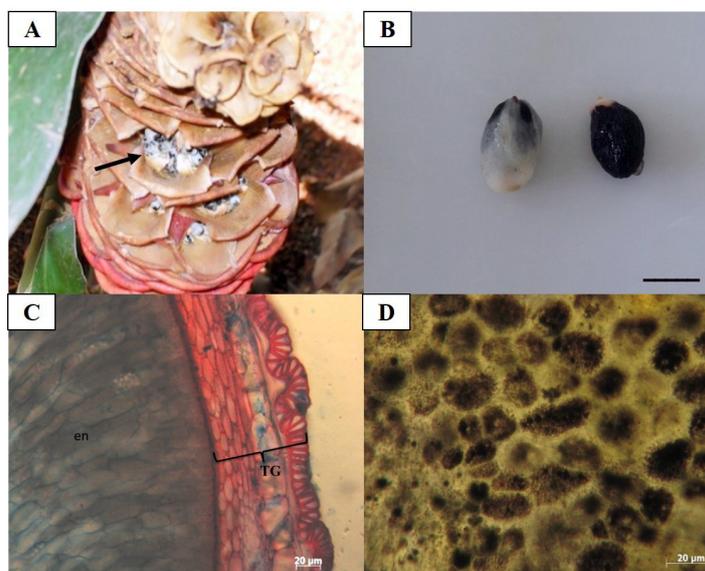
Figure 1. Inflorescences of *Z. spectabile* showing the seed arrangement (A); Seeds aspect (B); Micrograph of the cross section of *Z. spectabile* seed tegument (TG) endosperm (en) (C); Cotyledon tissue stained with lugol showing starch grains (D). (Bar=1cm).

Some species of the family Zingiberaceae rarely produced fruit, this can be incompatibility problems or absence of pollinator (CHOON et al., 2016; THOMAS et al., 2016). The presence of seed is not reported for most areas of *Z. spectabile* cultivation. Similar results are observed for other species of genus. The reproductive behavior of a population of three wild species of *Zingiber* sp. (*Z. meesanum, Z. nimmonii* and *Z. zerumbet*) was evaluated in different geographic region in the south of India. Where *Z. neesanum* and *Z. nimmonii* produced seeds in all the populations evaluated, but *Z. zerumbetc* produced seed only in three areas of ten evaluated (THOMAS et al., 2016).

Z. spectabile seeds are black, surrounded by a gelatinous white mass and aril (Figure 1B) and have 6.06 mm in length, 3.22 mm wide and 2.83 mm thick. The weight of one thousand seeds corresponded to 46.4 g.

The presence of aril is documented to other species of Zingiberales. The seeds of *Z. zerumbet* are ellipsoids, black and surround by aril (KOGA et al., 2016). Morphological aspects of *Heliconia velloziana* seeds were analyzed and the seeds were characterized as elliptical in shape and with a thin integument (SIMÃO and SCATENA, 2003).

In the integument of *Z. spectabile* seeds, there is a dense and thick sclerenchyma layer which we believe that acts as a barrier to the resumption of embryo growth during seed germination. The seed coat has two different layers of cell groups; the first one consists of narrow and long palisade cells, while the second group consists of irregularly shaped cells with thick walls and very large cells. The cells of the cotyledons are very bulky, with a great mass of starch grains (Figure 1C-D). In seeds of *Heliconia velloziana*, the endosperm occupied a large part of the seeds and contained a substantial quantity of starch and numerous spherical bodies, probably lipid deposits (SIMÃO et al., 2006).

The seeds had a moisture content of 22% at the time of collection. Seed imbibition was performed for approximately 230 hours, allowing the construction of the respective curve (Figure 2). The mass gain due to water uptake and imbibition curve of *Z. spectabile* seeds present a tree phase pattern. In phase I, water imbibition occurred during the first 10 hours, which suggests that the seed coat is permeable to water.
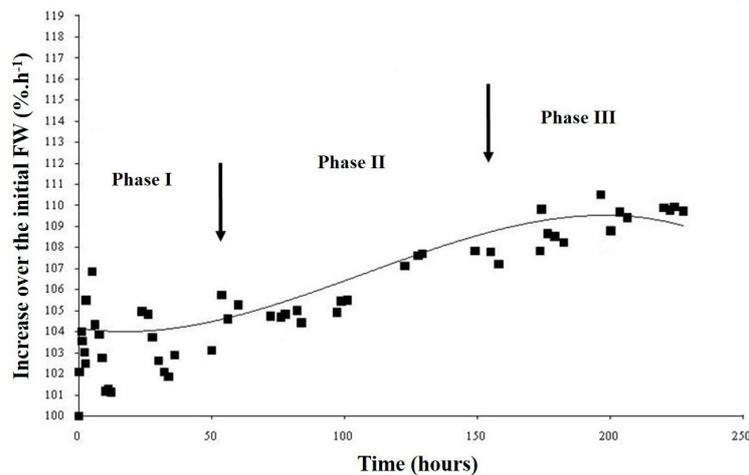
Figure 2. Increase over the initial fresh weight (% h^{-1}) of *Z. spectabile* seeds along imbibition period.

In phase II, the weight gain was low and continuous (61-158 hours), probably due to induced cellular osmotic potential since the matric potential became very limited. The absence of the stabilizing mass indicates that the osmotic potential was not completely nullified by the wall potential. Phase II is characterized by constituent activities of the preparatory biochemical process, being necessary for the enzymatic synthesis of DNA and mRNA depleted during Phase I (NERY et al., 2007). The beginning of phase III made visible the resumption of embryo growth, and was then identified by the protrusion of the primary root. Weight gain of *Zingiber spectabile* seed was found accelerated from 158 h, corresponding to the beginning of that stage.

The highest germination percentage (68%) and germination speed index (GSI, 0.25) were observed in seeds germinated at alternating temperatures of 20-30 °C (Table 1).

Table 1. Germination and GSI of *Z. spectabile* seeds at different temperatures

Germination (%)	Temperatures	
	20 ºC-30 ºC	**25 ºC**
	67.00 ± 3.52 [a]	53.0 ± 2.23 [b]
GSI	0.25 ± 0.01 [a]	0.19 ± 0.03 [b]

* Data: average ± standard error (SE)

Similar results were found for *Schizolobium parahyba* (SOUZA et al., 2012) and *Leucospermum* sp. (BRITS et al., 2014) where seeds incubated at alternating temperatures led to breaking of physical dormancy and earlier germination. Indeed, alternating temperature improves germination by breaking dormancy in some species (PROBERT, 2000). The temperature cycle characteristic of minimum, maximum and amplitude plays a role in signaling the seed for germination. By using alternating temperatures also during *ex vivo* germination, it is possible to simulate the diurnal fluctuation that occurs in the natural environment (BASKIN and BASKIN, 2001).

The germination of *Z. meesanum, Z. nimmonii and Z. zirumbet* seed was low, the average was below of 35% under continuous temperature of 18.16%, 23.16% and 34.33%, respectively (THOMAS et al., 2016).

The root protrusion of *Z. spectabile* seeds after 15 days, as well as seedling development are illustrated in Figure 3 A-D. *Heliconia velloziana* seed germination was after four month (SIMÃO and SCATENA, 2003). *Z. spectabile* seeds established in *vitro* had a fast germination and good seedling development. This can be because of starch content present in the seed (that improve the germination rate and seedling growth). The *in vitro* condition, with environment controlled is other factor that can improves de seedling development. The use of seed instead of the use of rhizome, most commonly used for this species, on *in vitro* establishment, is high recommended because seeds presents genetic diversity, sanity (seeds are a biological filter to diseases), and easier *ex vitro* (seed banks) and *in vitro* (cryopreservation) conservation.
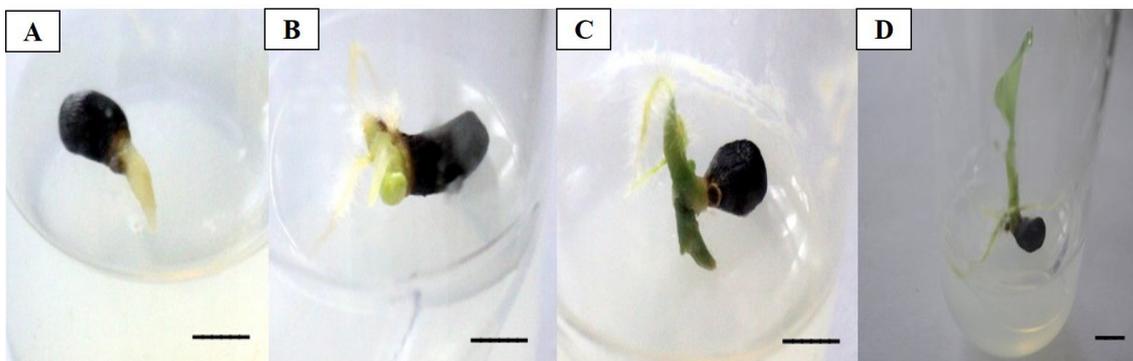
Figure 3. Aspects of seeds germination *in vitro,* with root protrusion and seedling development after different times of inoculation the *Z. spectabile* seeds. A= 15 days, B=20 days, C= 25 days and D= 30 days. (Bars=1 cm).

Effects of activated charcoal

No statistical differences were observed to shoot length, leaves number, root number, shoot number and fresh weight to different AC concentration. The AC concentration affected root length and senescent leaves number of the plantlets (Table 2). The highest root length was observed with the addition of 0.1% and 0.3% AC (Table 2).

Table 2. Effect of activated charcoal (AC) on shoot length (cm), root length (cm), number of leaves, senescent leave, shoot and fresh weight (g) of *Z. spectabile* plantlets.

AC	Shoot length (cm)	Root length (cm)	Leaves Number	Root Number	Senescent Leaves	Shoot Number	Fresh Weight (g)
0%	15.89 ± 0.59[a]	6.82 ± 0.67[b]	10.00 ± 0.42[a]	8.84 ± 0.29[a]	4.20 ± 0.44[a]	2.65 ± 0.39[a]	5.04 ± 0.42[a]
0.1%	17.22 ± 0.44[a]	8.67 ± 0.54[a]	10.45 ± 0.49[a]	9.00 ± 0.24[a]	3.45 ± 0.49[a]	2.90 ± 0.31[a]	5.09 ± 0.49[a]
0.3%	16.90 ± 0.17[a]	8.45 ± 0.42[a]	9.95 ± 0.37[a]	9.11 ± 0.33[a]	1.70 ± 0.46[b]	2.50 ± 0.33[a]	5.49 ± 0.37[a]

* Data: average ± standard error (SE)

Similar results were observed in palm *Phoenix dactylifera*, where the addition of AC led to higher length of roots (ABDULWAHED, 2013). However, in different genotypes of *Populus alba*, the use of AC increased root dry biomass and number of roots per shoot (DI LONARDO et al., 2013). One of the benefits of AC is creates a partial darkness of environment that is beneficial to root growth. A good root system development is important for a better establishment of plants and success during acclimatization.

It is well known that the inclusion of AC in the medium favours *in vitro* proliferation by reducing the exudates which inhibit growth. Also here, *Z. spectabe* plantlets benefited from the inclusion of AC in the growth medium, as it resulted with a decrease in the number of senescent leaves (Table 2). It is also important to consider the correlation between root length and senescent leaves.

Plants that showed the highest root length were the ones with lower number of senescent leaves. This can occur because of the good root absorption system, providing more nutrients. Other benefits related to the addition of AC are the increase and maintenance in pH levels during cultivation, that results in an uptake of more nitrogen and causes an improved growth besides the visual aspect of the explants (EYMAR et al., 2000).

Effect of sucrose concentration

The addition of sugar was not essential in initial stages for plantlet development of *Z. spectabile*. In the present study, the use of 0.3-0.7 M sucrose in medium resulted in reduced growth of plantlets (Table 3). This reduction may be due to an excessive osmotic potential induced in the medium.

Table 3. Effect of sucrose concentration on shoot length (cm), root length (cm), fresh weight (g), number of leaves, root and shoot of *Z. spectabile* seedlings.

Sucrose	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)	Leaves Number	Root Number	Shoot Number
0 M	11.14 ± 0.80[b]	4.45 ± 0.80[b]	1.06 ± 0.21[b]	4.55 ± 0.30[b]	5.45 ± 0.33[c]	0.00 ± 0.00[b]
0.1 M	15.70 ± 0.31[a]	7.63 ± 0.31[a]	1.94 ± 0.14[a]	6.60 ± 0.33[a]	10.1 ± 0.53[a]	0.00 ± 0.00[b]
0.3 M	7.13 ± 0.55[c]	4.65 ± 0.55[b]	1.91 ± 0.16[a]	5.60 ± 0.43[a]	8.35 ± 0.36[b]	4.40 ± 0.44[a]
0.5 M	5.89 ± 0.35[d]	3.44 ± 0.35[c]	0.70 ± 0.09[b]	4.35 ± 0.39[b]	4.75 ± 0.37[c]	0.60 ± 0.21[b]
0.7 M	5.50 ± 0.30[d]	3.41 ± 0.30[c]	0.44 ± 0.06[c]	3.60 ± 0.27[b]	3.57 ± 0.37[d]	0.21 ± 0.09[b]

* Data: averages ± standard error (SE).

The best plantlet elongation was obtained by use of 0.1 M sucrose. Plants showed the highest fresh weight (1.94 g), shoot length (15.71 cm), root number (10.10), root length (7.63 cm) and leaf number (6.6) by using 0.1 M sucrose. Shoot induction was affected by sucrose concentration. The highest values (4.4 shoots) were observed with 0.3 M sucrose (Table 3). *In vitro* propagation of plants is not fully autotrophic. Under these conditions, plants have low photosynthesis rate, and the addition of a carbon source

is necessary for the improvement of micropropagation efficiency.

Carbohydrates in culture media serve as energy carbon sources maintain the osmotic potential and are signaling molecules. This energy available from carbon sources is used in the developmental process (shoot proliferation, root induction and flowering), embryogenesis and organogenesis, which are highly energy demanding processes (YASEEN et al., 2013).

Effect of sealing system and culture medium type

There was no difference between the systems of flask sealing covers with or without filters at the center and culture medium type MS or WPM, regarding *Z. spectabile* growth during micropropagation processes.

Acclimatization

All plantlets survived the acclimatization process in commercial substrate after four week of transference to *ex vitro* environment. Similar results were also reported by Oliveira et al. (2010) during the acclimatization of *Z. spectabile,* with the use of other substrates and inoculated with fungus arbuscular mycorrhizal fungi. Others species of Zingiberales (*Etlingera elatior*, *Zingiber officinal, Heliconia velloziana*) also presented great survival to acclimatization process (GIRARDI and PESCADOR, 2010; DAS et al., 2013; QUISEN et al., 2014; RODRIGUES et al., 2015).

The acclimatization stage is very important to the success of utilization micropropagated plant to *ex vitro* cultivation. Thus, the good survive of *Z. spectabiles* acclimatized plants; indicate that large scale micropropagation of *Z. spectabile* can be use in the commercial cultivation of this species, providing healthy plants with phytosanitary.

4. CONCLUSIONS

The seeds of *Zingiber spectabile* have length of 6.06 mm, 3.22 mm width and 2.83 mm thickness. The weight of 1,000 seeds is 46.4 g. The *in vitro* establishment can be performed by using seeds, with better germination rate when alternating temperature from 20-30 ºC is used. Multiplication phase is optimized using 0.3% activated charcoal and 0.1 M sucrose. The micropropagated plants rate survival was 100% during the acclimatization process.

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AUTHORS CONTRIBUTIONS

M.V.R.: main author of the manuscript, in this way performing all analyzes, tabulation of data, and discussion of the results. **F.C.N.:** all analyzes of seed characterization and anatomy, writing and discussion of the results. **D.O.P.**: assistance in the implementation of tests on seeds, collection, tabulation and interpretation of data and construction of related tables and charts. **P.D.O.P.:** performing the experiments, writing and discussion of the results. **R.P.:** writing and discussion of the results. **R.T.F.:** multiplication of material and analyses of results. **D.P.C.S.:** statistical analysis and results discussion.

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