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Evaluation of new protocols to *Curcuma longa* micropropagation: A medicinal and ornamental specie

Meire Pereira de Souza Ferrari, Dirlane Antoniazzi, Andressa Bezerra Nascimento, Larissa Fiama Franz, Camila Silva Bezerra and Hélida Mara Magalhães*

Pós-graduação em Biotecnologia Aplicada à Agricultura, Pç. University Paranaense, Mascarenhas de Moraes, 4282, CEP 87502-210, Umuarama, Brazil.

Turmeric (*Curcuma longa*) is an Asian native species used for ornamental, medicinal and food purposes. However, its conventional propagation method is costly and inefficient. Therefore, this study aimed to verify appropriate asepsis methods for shoots from rhizomes, evaluate concentrations of activated charcoal and sucrose, and determine appropriate salt concentrations in Murashige and Skoog (MS) medium for turmeric growth. Three independent assays were performed. First, asepsis methods were verified for explants; second was for concentrations of sucrose and activated charcoal and in the final assay salt concentrations was evaluated. In all assays, MS medium supplemented with 30 g/L of sucrose, 6.5 g/L of agar and pH adjusted to 5.8 was used. Two growth regulators were added to the culture medium: 4.44 µM of 6-benzyl-amino-purine (BAP) and 1.08 µM of α-naphthalene acetic acid (NAA). Data obtained were subjected to analysis of variance (ANOVA) at $p \leq 0.05$ probability. The averages were compared by Tukey's test and polynomial regression at $p \leq 0.05$. All the asepsis methods evaluated showed similar effects. The highest concentration was obtained by utilizing 60 mg/L sucrose for the most part of the evaluated characteristics; however, it is recommended that activated charcoal be used at a concentration of 4.5 g/L. For salt concentrations, averages were compared by polynomial regression at ($p \leq 0.05$) probability in 50 to 60% MS medium, and resulted in longer root systems, greater numbers of shoots and more leaves. The usual dose of 3% sucrose resulted in lower development of *in vitro* seedlings. Salt concentrations higher than 60% were toxic to turmeric tissues and consequently compromised the root system and the aerial part of developing seedlings.

Key words: Turmeric, *Curcuma longa*, Zingiberaceae, culture medium, micropropagation.

INTRODUCTION

Turmeric (*Curcuma longa*), popularly known as turmeric, is a perennial herb from the Asian native Zingiberaceae family (Chakraborty et al., 2011). It is broadly used as a medicine because it is rich in compounds like curcumin,
sodium curcuminate and methyl-curcumin (Li et al., 2011; Ramadan et al., 2011) that have been studied due to their anti- inflammatory, antioxidant and antitumor potential (Chainani, 2003; Kainsa et al., 2012; Green and

Mitchell, 2014). It also has an antimicrobial effect against *Aspergillus* sp. fungi and bacteria from *Pseudomonas, Staphylococcus* and *Salmonella* genera (Rao and Mittal, 2014). In the cosmetics industry, it is used to produce body lotions and moisturizers (Gonçalves et al., 2014), whereas in the food industry, rhizomes are used to manufacture colorings that are usually used in pasta, in the preparation of sauces and breads (Shaw and Panda, 2015). It is also commonly used in landscaping projects for gardens and parks, and in flower commerce for floral arrangements and bouquets (Velayudhan et al., 2012).

In Brazil, turmeric has adapted very well to cultivation conditions. Nevertheless producers still lack information, mainly regarding to the cultivation methods and to varieties which may promote curcuminoids increment and starch production, once this specie has expressive amount of this type of carbohydrate (76 and 86, 62%) (Santana and Meireles, 2014) end in particular regarding the initial phase of plant propagation (Berni et al., 2014).

Currently, the rhizome propagation method is conventionally used (Berni et al., 2014). Rhizomes are usually collected and re-planted in October in most of the country due to their dormancy and the beginning of the rainfall season. This technique presents some limiting factors for seedling production, such as cost, production time, labor, and utilization of the commercialized part the rhizomes (Miachir et al., 2004). The contamination of the propagation material with soil fungi, bacteria and nematodes present in soil (Faridah et al., 2011) is another obstacle that results in loss of the propagation material. Moreover, a rhizome has low yield because it propagates, at most, four seedlings.

One way to relieve these limiting factors for turmeric establishment may be through the culture medium in vegetal tissues. This technique obtains desirable agronomic characteristics, such as pathogen-free plants, abundant and fast seedling production throughout the year (Sidhu, 2010; Ahmadian et al., 2013). For example, one single explant can result in up to 300 seedlings, with large-scale production of excellent quality material (Yildiz, 2012). *Etlingera elatior* seedlings were capable of forming at the end of 30 days from 8 to 10 shoots in MS medium (Colombo et al., 2010). In addition, homogenous seedlings usually reach physiological maturity faster in the field, with shorter plant juvenility and an earlier production start (Souza et al., 2011).

However, for the micropropagation of vegetable material, it is necessary to develop the appropriate culture medium for each species (Sridhar and Aswath, 2014). This culture medium should consist of ideally balanced compounds in order to favor morphogenesis and produce vigorous plants (Yildiz, 2012; Ahmadian et al., 2013). Several aspects must be standardized in the culture medium such as asepsis, salt concentration, carbon sources, antioxidants, pH and growth regulators. The latter are mostly verified in assays involving tissue culture. However, aspects like asepsis, salt concentration,

and carbon and antioxidant content should not be neglected. Therefore they should be investigated to standardize protocols because inappropriate nutrition can cause difficulties in seedling growth (Sridhar and Aswath, 2014).

Nevertheless there is lack of information about culture medium constituents for turmeric, and research results have often been inconclusive. According to Babaei et al. (2013), asepsis is the most limiting phase of the micro-propagation process, mainly due to the bacteria from *Bacillus*, *Pseudomonas*, *Staphylococcus* and *Lactobacillus* genera and fungi from *Aspergillus* sp. *Penicillium* spp. and *Fusarium* spp. Genera, which are among the most commonly found contaminants that cause explant death and serious economic, propagating material and time losses over the micropropagation process (Sidhu, 2010; Yildiz, 2012). The use of disinfecting products in the sterilization process of several species Zingiberaceae is essential because explant sources in assays are axillary buds obtained for example from rhizomes or shoots of philippine wax flower and ginger (Colombo et al., 2010; Abdelmageed et al., 2011; Faridah et al., 2011). Rhizomes, which are underground materials, are exposed to a great variety of pathogens, such as fungi and bacteria (Rescarolli and Zaffari, 2009), making explant establishing in the initial cultivation phase difficult. As is the case with rhizome and root rotting caused by (*Rhizoctonia solani* and *Fusarium oxysporum* f. sp. cubense) at *E. elatior* and *Heliconia chartacea* cv. Sex Pink. and nematodes from Meloidogyne, Radopholus and Helicotylenchus species and genus (Lins and Coelho, 2004).

Salt concentration is another important factor as media with usual concentration fare (100%) is not always ideal in a micropropagation protocol. Excessive salt can cause the opposite effect, limiting the development of roots and aerial parts. Moreover, some species such as *Ocimum basilicum* are frequently intolerant to high salt concentrations in the range of (70 to 100%) (Tarchoune et al., 2012). Species with concentration lower than 70% have been more favorable to the development and with regard to *Solanum tuberosum* where 50% of the MS medium salts caused vigorous and health seedlings (Bandinelli et al., 2013). As reported by Islam et al. (2004), a 75% salt concentration was more important for induction of microrhizomes in turmeric than was treatment with 100% salt concentration.

Activated charcoal is the most common antioxidant used to absorb unwanted compounds from the medium (Thomas, 2008). However, it also absorbs growth regulators, vitamins, copper and zinc. Therefore the previous standardization of activated charcoal is recommended (Thomas, 2008; Ahmadian et al., 2013). Its beneficial effect was verified in *Strelitzia reginae*, which phenolics were reduced at 53% in the medium, when compared with media supplemented with ascorbic acid (North et al., 2012). Sucrose is generally used as the

Table 1. Asepsis methods used for *Curcuma longa* shoots kept under *in vitro* conditions.

Treatments (t)	Description
1	Immersion in sodium hypochlorite solution at 2% (v v[-1]) for 20 min, followed by washing with deionized water and autoclaved for three times
2	Similarly as T1–time increased to 40 min in sodium hypochlorite solution at 2% (v v[-1]), followed by triple washing
3	Immersion in fungicide: 100 µl/100 ml of Rovral® water[1] 100 µl/100 ml of Locker® water[2] 100 µl/100 ml of Priori xtra®water[3]. Shoots were left in fungicide for 30 minutes and another 20 min in sodium hypochlorite at 2% (v v[-1]), followed by triple washing
4	As for T3, but with time increased to 60 min in fungicide and another 20 min in sodium hypochlorite at 2% (v v[-1]),followed by triple washing.
5	Immersion in fungicide: 300 µl/100 ml of Rovral® water[1] 300 µl/100 ml of Locker® water[2] 300 µl/100 ml of Priori xtra®water[3], left for 30 min in fungicide and another 20 min in sodium hypochlorite at 2% (v v[-1]),followed by triple washing
6	As for T5, but with time increased to 60 min in fungicide and another 20 min in sodium hypochlorite at 2% (v v[-1]),followed by triple washing

[1]Rovral®-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxo imidazoline-1-carboxamide (IPRODIONE). [2]Locker® - Carbendazim 200 g/L + Tebuconazole 100 g/L + Kresoxim-methyl 125 g/L. [3]Priori xtra® - Azoxystrobin 200g/L + Cyproconazol 80 g/L.

carbon source at 3% concentration in MS medium (Yassen et al., 2013), but higher concentrations provided greater growth of aerial parts and roots, which is beneficial over the micropropagation process once seedling may develop faster over a short period in the laboratory. Some studies have shown that species of the Zingiberaceae family respond favorably to increases in sucrose concentration from 3 to 6% (Islam et al., 2004; Kusumastuti et al., 2014). This has been observed for *Curcuma aromatica*, which showed 70% more rooting at 6% concentration when at the usual 3% concentration (Nayak, 2000). Other Zingiberaceae species enhanced due the higher concentrations of sucrose from were *C. Longa* with 6% (Jala 2012), *Curcuma xanthorrhiza* with 9% (Kusumastuti et al., 2014) and *Zingiber officinale* 8% of sucrose (Singh et al., 2014).

Therefore, this study aimed to verify appropriate asepsis methods for shoots from rhizomes, evaluate concentrations of activated charcoal and sucrose, and verify salt concentrations in Murashige and Skoog (MS) medium in the growth of turmeric.

MATERIALS AND METHODS

Turmeric rhizomes were obtained from *Cooperativa dos Produtores de Açafrão* Mara Rosa - Goiás, Laboratory of Molecular Biology and Culture of Vegetal Tissues from Paranaense University (UNIPAR, Umuarama PR, Brazil). Cracked, withered rhizomes with disease symptoms were discarded and only the most vigorous rhizomes were selected. These were kept in laboratory ambient conditions without temperature and moisture control until the start of the experiments. Shoots from the rhizomes were used for assay installation and were removed with a scalpel. The shoot length was standardized to approximately 1.5 cm ± 0.3.

Shoot asepsis

To evaluate asepsis methods, shoots previously obtained were submitted to six different procedures as described in Table 1.The procedures were carried out in a laminar flow hood. Shoots from the asepsis step were inoculated in 350 ml clear glass flasks containing MS culture medium (Murashige and Skoog, 1962), supplemented with 30 g/L of sucrose and 6.5 g/L of agar. Two growth regulators of 4.44 µM of 6-benzyl-amino-purine (BAP) and 1.08 µM of α-naphthalene acetic acid (NAA) were added to the culture medium (Prakash et al., 2008). The pH was adjusted to 5.8 and then sterilized by autoclaving at 121°C for 20 min. Inoculation was carried out in an aseptic chamber. The shoots were individually displayed in flasks containing 50 ml of culture medium, closed with transparent plastic lids, and sealed with polyvinyl chloride (PVC).

The material was then kept in a growth room for 90 days at a temperature of 25 ± 2°C, and submitted to light intensity of 60 µmoles/m^{2}/s and 24-h light photo periods. The evaluated parameters were: the contamination total fungal and bacterial (expressed as percentage), and explant oxidation (expressed as percentage; number of leaves; length of aerial part and root (mm);

Table 2. Percentage of contaminated seedlings (CON), oxidated seedling (OXD),number of leaves (NL), aerial part length (APL), base diameter (BD), fresh mass of aerial part (FMAP), fresh mass of roots (FMR), dry mass of aerial part (DMAP), and dry mass of root (DMR) of *C. longa* in function of different asepsis methods after 90 days of *in vitro* cultivation.

Treatments	CON (%)	OXD (%)	NL	APL (mm)	BD (mm)	FMAP (g)	FMR(g)	DMAP (g)	DMR (g)
1	0±00[a]	12.50±2.12[a]	5.00±0.17[a]	95.116±3,00[a]	5.169±0.28[a]	2.376±0.38[a]	1.939±0.38[a]	0.686±0.01[a]	0.174±0.007[a]
2	6.250±1.41[a]	50.00±1.520[a]	3.93±0.20[a]	96.566±1.22[a]	35.427±1.02[a]	0.725±0.07[a]	2.559±1.22[a]	0.177±0.005[a]	0.066±0.000[ab]
3	12.500±2.12[a]	31.25±1.73[a]	3.50±0.23[a]	70.780±0.14[a]	5.055±0.38[a]	1.219±0.15[a]	1.782±0,14[a]	0.126±0.004[a]	0.054±0.002[ab]
4	12.500±2.12[a]	6.25±1.41[a]	4.12±0.39[a]	127.819±0,14[a]	4.085±0.94[a]	1.496±0.11[a]	1.844±0.14[a]	0.153±0.006[a]	0.043±0.006[b]
5	6.250±1.41[a]	18.75±0.7[a]	4.58±0.40[a]	75.004±0.55[a]	6.285±0.04[a]	0.863±0.07[a]	2.598±0.55[a]	0.141±0.008[a]	0.041±0.007[b]
6	18.750±0.7[a]	18.75±0.7[a]	5.04±0.11[a]	86.874±0.11[a]	8.290±0.58[a]	1.608±0.09[a]	2.513±0.10[a]	0.139±0.012[a]	0.029±0.001[b]

* Means with the same letters in the same column are not significantly different at p ≤0.05. Note: cont (%) and oxd (%) analyzed by Kruskal-Wallis's test, test value=5.18, standardized value =14.24

longitudinal diameter of the base (mm), measured by a digital pachymeter; and dry and fresh matter of aerial part and root. To measure fresh matter, fresh seedlings were weighted in an analytical balance; dry matter of the vegetable material was separated into paper bags and subjected to an air circulation oven at 65°C for 4 days until it reached constant weight.

The experimental design performed in this work was a completely random design with six treatments, five replicates, and four flasks per plot, totaling 120 plots. Contamination data and oxidation percentage were analyzed with non-parametric analysis using a Kruskal-Wallis' test at p ≤ 0.05 of probability. The remaining data were analyzed using analysis of variance (ANOVA) at p ≤0 .05. Averages were compared by Tukey's test at p ≤ 0.05 of probability, using the SISVAR program (Ferreira, 2011).

Effect of activated charcoal and sucrose

The effect of activated charcoal and sucrose on the development of turmeric was evaluated at Step 2. Shoots were sterilized with sodium hypochlorite (2%) in a laminar flow chamber for 20 min, then washed with deionized water and autoclaved for three times, according to the results of essay 1. After asepsis, the shoots were inoculated in a culture medium prepared using the protocol described elsewhere (Prakash et al., 2008). The characteristics evaluated were: total fungal and bacterial contamination (expressed as percentage), explant oxidation (expressed as percentage), length of aerial part and root (mm); base longitudinal diameter (mm) which was measured by a

digital pachymeter, dry and fresh matter of aerial part and roots, and number of shootings and leaves. The experiment was a completely random design in a factorial scheme (2 × 3) with two levels of sucrose concentrations (30 and 60 g/L), and three levels of activated charcoal concentrations (0, 3.0 and 4.5 g/L), and three levels of activated charcoal concentrations (0, 3.0 and 4.5 g/L). A total of six treatments with five replicates and four flasks per plot were prepared. The data were subjected ANOVA at p ≤ 0.05 and the means were compared using Tukey's test at p ≤ 0.05, using SISVAR program (Ferreira, 2011).

Salt concentration

The ideal salt concentration for *in vitro* cultivation of turmeric was tested. Shoots were sterilized with sodium hypochlorite (2%) for 20 min in a laminar flow hood, then washed in deionized water and autoclaved three times according to the results of essay 1. The culture medium was prepared according to Prakash et al. (2008). The same characteristics as for previous assays were evaluated: total fungal and bacterial contamination (expressed as percentage), explant oxidation (expressed as percentage, length of aerial part and root (mm), base longitudinal diameter (mm), measured by a digital pachymeter, dried and fresh matter of the aerial part and root, and the number of shootings and leaves. A completely random design was applied to the experiments. The evaluated salt concentrations in MS medium were: 0, 25, 50, 75 and 100%. Five treatments, five replicates, and four flasks per plot were performed. Results were subjected to ANOVA at p ≤ 0.05. The means were

compared by polynomial regression at p ≤ 0.05, using the SISVAR software (Ferreira, 2011).

RESULTS AND DISCUSSION

Shoot asepsis

The influence of shoot asepsis resulted in statistical differences among treatments for root dry matter (Table 2). Treatment 1 showed the greatest weight average.

Treatments 5 and 6 showed the same average results for root dry matter, which were both greater than that resulting from treatment 2. Overall, it was observed that for some species of the Zingiberaceae family, the highest explant contamination occurred in buds from ginger rhizomes (Faridah et al., 2011), chinese ginger (Yusuf et al., 2011) and mango ginger (Mohanty et al., 2012).

Rhizomes are underground stems in direct contact with soil microorganisms, including pathogens like bacteria and fungi. This probably contributed to a greater infection of the buds and consequently affected the contamination rate at the time of *in vitro* establishment. For other studies that evaluated asepsis in Zingiberaceae

Table 3. Average values of isolated effect of sucrose concentration for base diameter (BD), fresh matter of aerial part (FMAP), fresh matter of root (FMR), dry matter of aerial part (DMAP) and dry matter of root (DMR) of *C. longa.*

Treatment	BD (mm)	FMAP (g)	FMR (g)	DMAP (g)	DMR (g)
30 g/L	5.90±0.50[b]	0.19±0.18[b]	8.68±0.17[a]	0.15±0.05[b]	0.15±0.03[b]
60 g/L	7.68±1.9[a]	0.70±0.00[a]	2.90±0.77[b]	0.30±0.04[a]	0.65±0.11[a]

*Means with the same letters in the same column are not significantly different at p ≤0.05.

Table 4. Average values of isolate effect of activated charcoal concentration for base diameter of *C. longa* seedlings.

Treatment activated charcoal	Base diameter (mm)
0.0 g/L	5.37 ±0.94[b]
3.0 g/L	5.49 ±0.80[b]
4.5 g/L	9.49 ±0.73[a]

*Means followed by the same letter do not differ among themselves by Tukey's test at (p≤0.05).

buds, stronger substances were required, such as ethanol immersion (70%) for 2 min and two drops of mercury chloride solution at 0.1 (%) in turmeric (Islam, 2004), and mercury chloride solution at 0.1 (%) in wild turmeric (Nayak, 2000). Clorox at 60% was used for 30 min on bitter ginger, then all bud external tissues were removed and the explants were again subjected to asepsis with 20% of Clorox (Faridah et al., 2011).

In this study, shoots from rhizomes but not from buds were used and, probably due to the meristematic origin and adjacent leaf tissues at the borders of the leaves, these factors contributed to a greater protection against pathogens, resulting in less tissue infection (Nair, 2013). Therefore the usual treatments using tissue culture with sodium hypochlorite (at 2%) was sufficient to sterilize explants and also caused less damage to the root system. However, when propagules originated from rhizomes (buds), stronger disinfecting substances like fungicides and mercury chloride should be used for asepsis as used for ginger (Faridah et al., 2011), Chinese ginger (Yusuf et al. 2011) and mango ginger (Mohanty et al., 2012). Although this type of treatment is needed in these cases, it is time consuming and requires significant effort.

Effect of activated charcoal and sucrose

There was an interaction effect between activated charcoal and sucrose concentrations for the factors evaluated in this work (Table 5). For the other assessed characteristics, such as fresh and dry matter of aerial part and root and base diameter, only the isolated effects of sucrose (Table 3) and activated charcoal (Table 4) were observed. It was verified that sucrose solution at 60 g/L favored almost all evaluated characteristics. Only fresh matter of the root was not increased by this treatment

(Table 3). The best concentration for activated charcoal was 4.5 g/L. This treatment showed an increase of 4.08 mm of the base diameter when compared to the control sampling. For the usual concentration of sucrose (3.0 g/L), the increase was 4.00 mm. There were no statistical differences between the control and 3.0 g/L of activated charcoal concentration (Table 4).

Sucrose is the most utilized carbon source in most protocols (Ahmadian et al., 2013). Sugars are necessary for the function of several parts of morphogenesis, such as the osmolarity balance of the culture medium, skeletal function for the building of various important factors, heterotrotophy/mixotrophy, and regulation of genes responsible for plant growth (Yaseen et al., 2013). Some studies on plants from the Zingiberaceae family have shown that higher sucrose concentrations of 60 to 70 g/L are more efficient for *in vitro* plant morphogenesis when compared to the usual concentration of 30 g/L. This was verified in assays carried out with wild turmeric in which rooting increased by 70% when compared to the usual concentration (Nayak, 2000). Moreover, at 60 g/L of sucrose in turmeric, a greater number of shoots, number of leaves, and root length (Jala, 2012) were observed.

Beyond sucrose, other carbon sources have been used in the micropropagation, as is the case of sorbitol, mannitol, fructose, galactose (pentoses) and maltose (disaccharide) (Yaseen et al., 2013). Being the first two, more are used in essays involving minimal cultivation or in germplasm collection establishment (Martins et al., 2011). The others may be used in future researches aiming to minimize production costs and the better seedlings development. Few essays evaluated the effect of other sugar sources compared to Zingiberaceae. A comparative study of wild turmeric seedlings cultivated in sucrose, glucose and maltose demonstrated that sucrose was the best source in relation to number of shoots and the length of aerial part (Sharmin et al., 2013). Jalil et al.

Table 5. Average values of sucrose and activated charcoal interaction for contamination percentage (CP%), number of shoots (NS), length of aerial part (LAP) and length of root (LR) in mm of *C. longa*.

Charcoal g/L	Sucrose g/L		Sucrose g/L		Sucrose g/L		Sucrose g/L	
	30	60	30	60	30	60	30	60
	CP %		NS		LAP (mm)		LR (mm)	
0.0	10±0.70[Aa]	10±0.70[ABa]	1.30±0.23[Aa]	1.42±0.11[Ba]	93.25±1.94[Aa]	45.42±2.84[Bb]	59.45±1.60[Aa]	48.92±0.86[Ba]
3.0	0±0.00[Ab]	30±1.41[Aa]	0.84±0.09[Aa]	1.15±0.10[Ba]	86.14±1.80[Aa]	86.14±1.37[Aa]	73.89±0.01[Ab]	121.01±1.44[Aa]
4.5	0±0.00[Aa]	5±0.70[Ba]	0.90±0.19[Ab]	2.45±0.06[Aa]	102.66±1.46[Aa]	102.66±0.76[Aa]	56.96±2.74[Aa]	21.42±2.19[Bb]

*Means followed by the same capital letter in the column and small letter in the line do not differ among themselves by Tukey's test (p≤0.05).

(2015) also verified that sucrose enhanced cells growth in a ginger suspension when compared with maltose, glucose and fructose. It is important to emphasize that these results corroborate the present study for the same evaluated characteristics, showing that plants from the Zingiberaceae family tend to respond positively to an increase in sucrose concentration in the culture medium. Different results were obtained for two species of *S. tuberosum*, which increase in sucrose concentration limited the number of leaves and the length of the aerial part of *in vitro* seedlings (Bandinelli et al*.,* 2013). In *Orbignya oleifera* embryos, the interaction between sucrose and activated charcoal was not significant. For the assessed characteristics, only the carbohydrate isolated effect was effective for concentrations of 34.5 g/L (Leite et al., 2014). This showed that the response to carbohydrate increase does not depend only on sugar and antioxidant interaction, but also on the genetic plant constitution.

The observed response for contamination percentage, number of shoots, and length of aerial part and root depended on the concentrations of sucrose and activated charcoal together (Table 5). The highest contamination percentage was observed at concentrations of 60 g/L of sucrose and 3.0 g/L of activated charcoal, followed by concentrations of 30 and 60 g/L of sucrose in the absence of activated charcoal 0.0 g/L. There were no contaminated explants at concentrations of 3.0 g/L of sucrose, and 3.0 and 4.5 g/L of activated charcoal. However, the treatment that showed most increase in the number of shoots was 60 g/L of sucrose and 4.5 g/L of activated charcoal. The activated charcoal concentration provided the same independent effect as sucrose concentration when compared to other treatments (Table 5). The growth of the aerial part was most effective in the medium with 30 and 60 g/L of sucrose and 3.0 and 4.5 g/L of activated charcoal. Nevertheless no statistical differences were apparent between the treatments. The smallest growth was observed for the treatment with 60 g/L of sucrose in the absence of activated charcoal. The results for root growth were similar to those described previously, except for the treatment with 60 g/L of sucrose and 4.5 g/L of activated charcoal, which was ineffective (Figure 1A to D). The growth was 5.6 mm smaller than the best treatment (60 g/L of sucrose and 3.0 g/L of activated charcoal) about 17.70% of lower root growth (Table 5).

The use of higher concentration of sucrose (60 g/L) does not necessarily require the use of a greater amount of activated charcoal, as the lengths of the aerial part and root suffer damages in the presence of high sucrose concentrations and absence of activated charcoal. Increase in temperature causes Mailard reaction, which occurs when the sucrose is submitted to high temperatures during the autoclave process and results in the carbohydrate partially hydrolyzing into glucose and fructose. In addition, at the end of the process, it forms a compound known as 5-hidroxymethyl-furfuraldeid, which is toxic to the vegetal tissue (Shibao and Bastos, 2011).

Moreover, when tissue is cut, it can release phenolic compounds that are also undesirable in morphogenesis, as they create polyphenolo-xydase (PPO), which uses ortho-diphenol com-pounds found in a cell vacuole substrate. Finally, when there is a cut in the tissue, quinone polymerization occurs since the substrate is in contact with an enzyme polyphenoloxidase in the presence of oxygen (Thomas, 2008). For example, catechins found in cocoa (Rusconi and Conti, 2010), gallic, cinnamic and caffeic acids were pointed out as substrate to polyphenoloxidase enzyme. These compounds were found in ginger (Ghasemzadeh et al., 2010; Siddaraju et al., 2011). Li et al. (2011) reported at least nineteen different types of curcumins in turmeric as potential substrate to PPO. Quinones and 5-hydroxymethyl-furfuraldeid can be extremely harmful to vegetal development.
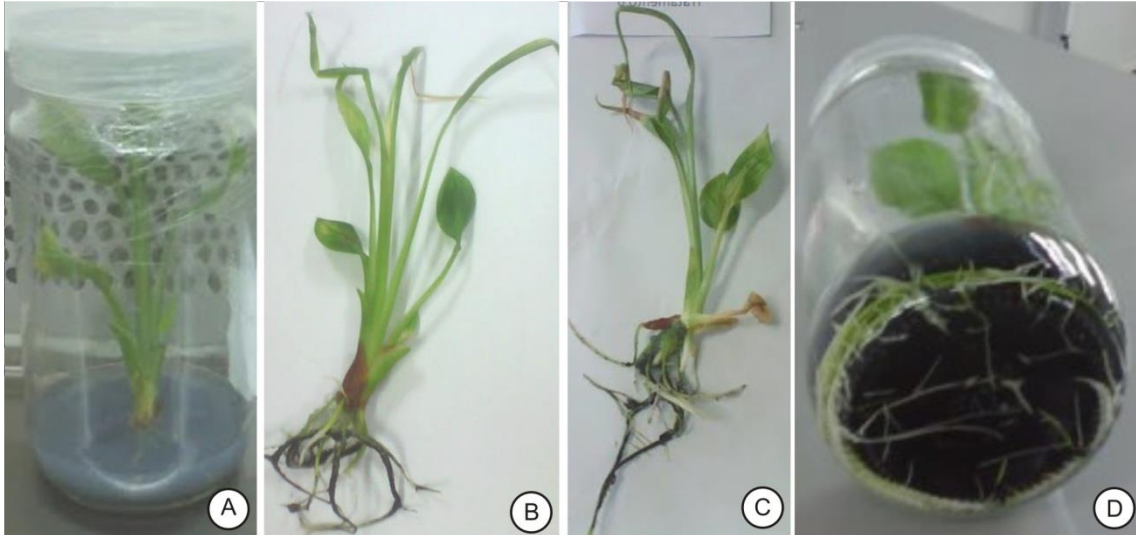
Species from Zingiberaceae family have the

Figure 1. *In vitro* seedlings of *Curcuma longa* in function of the concentrations of sucrose and activated charcoal. A) and D) Inoculation with (60 g/L) of sucrose and (4.5 g/L) of charcoal; B) Inoculation with (30 g/L) of sucrose and addition of (4.5 g/L) of charcoal; C)Inoculation with (60 g/L) of sucrose and addition of (3.0 g/L) of charcoal.

capacity to respond to a higher concentration of sucrose. It was evident in this study that activated charcoal was essential to the utilization of higher sucrose concentrations. This is probably the case because charcoal adsorbs more substances from the culture medium as it is formed by graphically arranged carbons with a thin pore network (Thomas, 2008). However, it is important to establish optimal charcoal concentration because it can also absorb growth regulators, vitamins, and ions such as copper and zinc.

Salt concentration

The salt concentrations affected the evaluated characteristics during the assay, with statistical differences observable at $p \leq 0.05$ (Figure 2). Contamination percentage, oxidation, base diameter, fresh matter of aerial part, and root dry matter were not influenced by salt concentrations in MS medium, and the same effects were observed in all evaluated concentrations.

The quadratic model was used for the number of leaves and shoots. It was observed that, initially, there was an increase in the number of shoots, up to salt concentration of 50%, after which a decrease occurred (Figure 2A), similarly as *Zantedeschia albomaculata*, which presents a greater number of shoots in MS medium at 50% of salt concentration (Chang et al., 2003). The salt concentration at 64.24% influenced the increase in the number of leaves, which was approximately five times greater than the control, as described on (Figure 2B). The length of root and aerial

part was better evaluated by the quadratic model. The best concentration for the length of aerial part was 64.81%. At higher concentrations, there was a decrease in approximately 25 mm for this characteristic (Figure 2C). There was an increase in root length at 48.59% concentration with posterior decrease, reaching approximately 20 mm for the highest concentration (100%), according to the (Figure 2D).

The quadratic model described the root fresh mass, and the best average masses were obtained at 60.56%. At higher concentrations, there was a reduction of approximately (3.0 g) of fresh mass. Compared to the control, the fresh mass gain was 4.5 greater (Figure 2E). The linear model was the best for dry matter of aerial part, therefore, the higher the salt concentration, the greater the gain for this characteristic (Figure 2F).

Most studies have shown that higher salt concentrations provide more severe damage to the root system. This seems obvious since the root is responsible for the absorption of ions (macro and micro nutrients) in ideal amounts (George et al., 2008; Khenifi et al., 2011). The same is true for *in vitro* cultivation. In our study, the increase in salt concentration also compromised the number of shootings and leaves. In the same way root system of *Butia capitata* was also damaged at 100% salt concentration and the best root development occurred at 75% of salt concentration (Ribeiro et al., 2011). Erig et al. (2004) observed a greater average length for *Cydonia oblonga* roots using 75% salt concentration in MS culture medium in accordance with Leitzke et al. (2009) who also stated that 75% of salt concentration in culture medium is ideal for *in vitro* rooting for the *Rubus fruticosus* ('Xavante' blackberry), producing a greater average
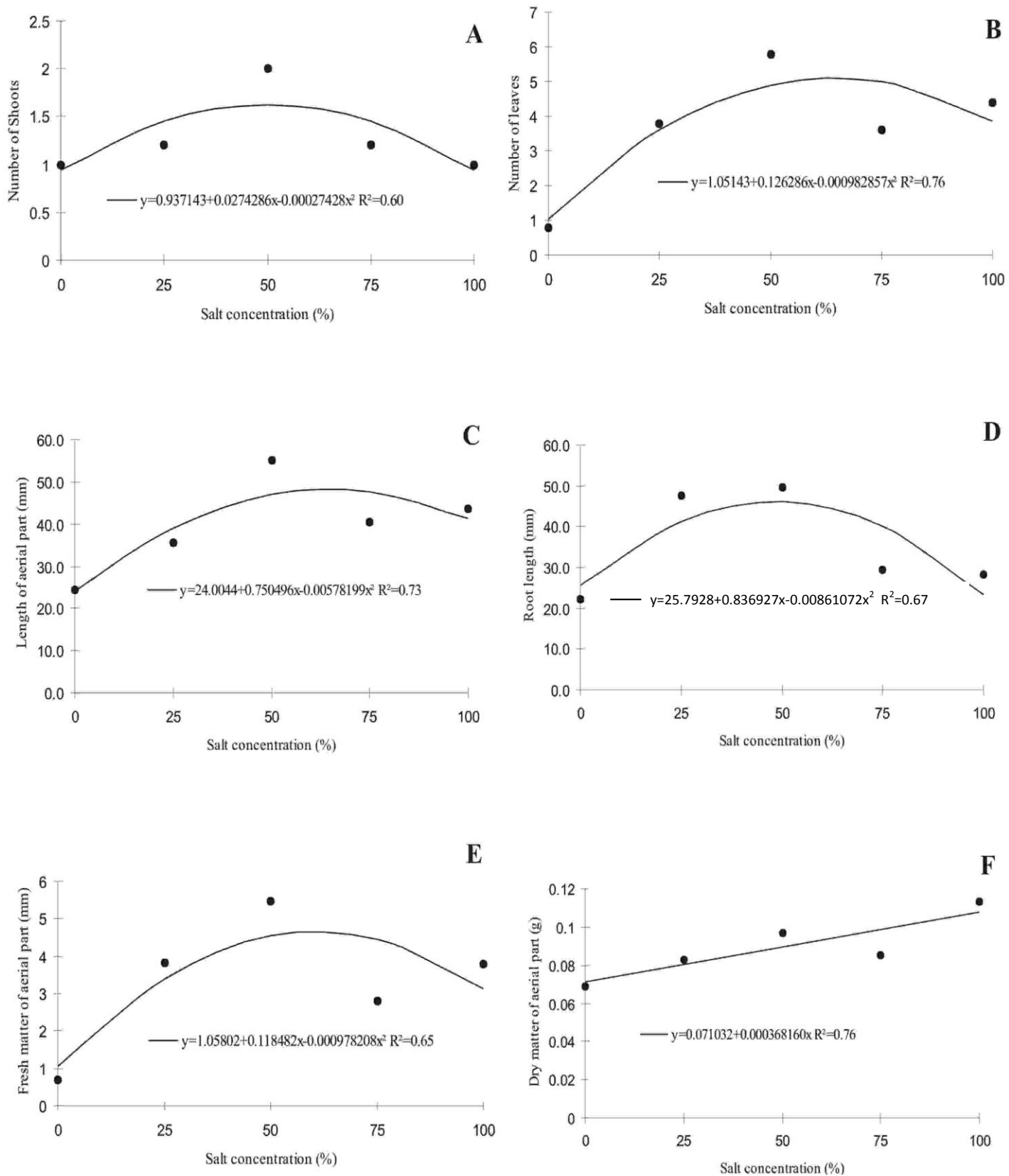
Figure 2. Number of shootings (A); Number of leaves (B); Length of aerial part (C); Root length (D); Root fresh matter (E); Dry matter of aerial part (F). All the characteristics were evaluated at different saline concentrations in Murashige and Skoog (MS) medium after 90 days. Black spots represent the mean values observed.

length of roots.

Ion absorption depends on synergisms and antagonisms. For example, when synergism occurs, the presence and amount of anions may favor the absorption of other anion through the roots. In antagonism the same ion can compete for the same absorption site, limiting the absorption (Silvia and Trevizam, 2015). These equilibrium dynamics may have occurred in the highest salt concentrations for turmeric medium. High concentrations of dissolved phosphate may reduce growth, probably due to the reduced absorption of elements such as Zn, Fe, and Cu. High amounts of K^+ or Ca^{2+} and Mg can result in the decrease of phosphate absorption if the solution pH becomes slightly alkaline (George et al., 2008). One way to maintain pH in the culture medium is through the dynamic equilibrium of ammonium and nitrate absorption. Quick ammonium absorption makes the medium pH decrease to 4.2 to 4.6. When this happens, ammonium capture is inhibited, but nitrate ion absorption is stimulated, causing the pH to increase again (George et al., 2008).

In addition, ammonium concentration may have been toxic to turmeric seedlings due to nitrogen in the form of ammonium nitrite (NH_4NO_3) in MS culture medium. Plants absorb nitrogen as nitrate (NO_3^-) and ammonium (NH_4^+) (Mokhele et al., 2012). The latter is very toxic to vegetal tissues if not assimilated (Britto and Kronzucker, 2002). Plants avoid toxicity from the rapid conversion of ammonium into amino acids (Mokhele et al., 2012). At high concentrations, this ion dissipated protons that are needed for electron transport in photosynthesis and respiration, and metabolites are capture in a vacuole (George et al., 2008). In *Arachis hypogaeaa*, the presence of ammonium over a 25:75 ratio (NH_4^+:NO_3^-) damaged the plant's initial development, but the combination of NH_4^+ and NO_3^- in 25:75 and 0:100 ratios provided greater phytomass production in this species (Ribeiro et al., 2012).

For turmeric, the ideal salt concentration range varied from 50 to 60%, in which the final concentrations of the culture medium for ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) were 825 to 990 mg/L and 950 to 1140 mg/L, respectively. The reduction of ammonium ion concentration (NH_4^+) in the medium contributed to smaller toxicity in the root system of turmeric. This toxicity may have contributed to a decrease in the number of leaves and shoots of the species.

Conclusion

All evaluated asepsis methods presented the same effect on the evaluated characteristics, except for the root dry matter. In that case, the usual asepsis method can be the most effective because it is less costly and demands less time. Therefore, it is recommended that shoots be washed in 2% sodium hypochlorite solution for 20 min, followed by three washings with deionized water. The concentration of 60 g/L of sucrose was the most feasible for turmeric. Nevertheless activated charcoal must be added to MS medium at 4.5 g/L. Although charcoal did not have an effect on all evaluated characteristics, it can be added because it did not compromise seedling development. Salt concentration in MS should range from 50 to 60% of the total amount of salts. When utilized at the highest concentration of 100%, it damages the root system, number of leaves, and shoots.

Conflict of Interests

The authors have not declared any conflict of interest.

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